Novel Signaling Function of hCLCA1 in Airway Macrophage Activation

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ABSTRACT

The *CLCA* gene family produces both secreted and membrane-associated proteins that modulate ion-channel activity, drive mucus production and have a poorly understood pleiotropic effect on airway inflammation. The mechanism of how hCLCA1 regulates airway inflammation remains unknown. However, *hCLCA1* induction in inflamed airway epithelium is higher than most cytokines and chemokines that activate resident and recruited macrophages' innate immune response. Thus, it is possible that hCLCA1's role in airway inflammation is to regulate the immune response of macrophages. Here, we demonstrated the ability of secreted hCLCA1 to activate macrophages, inducing them to express pro-inflammatory cytokines and to undertake a pivotal role in airway inflammation. The VWA domain within hCLCA1 was found to be responsible for the activation, which correlated with induction of the NF-κB and MAPK pathways. These findings open a new area of investigation into the function of CLCA proteins and should be of therapeutic interest.

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LIST OF ABBREVIATIONS

APC	-	Antigen-presenting cell
ASL	-	Airway surface liquid
ATT	-	Alpha 1-antitrypsin
BAL	-	Bronchoalveolar lavage
BME	-	β-mercaptoethanol
CaCC	-	Calcium-activated chloride channel
cAMP	-	Cyclic adenosine monophosphate
CF	-	Cystic fibrosis
CFTR	-	Cystic fibrosis transmembrane conductance regulator
CLCA	-	Chloride channel accessory protein
COPD	-	Chronic obstructive pulmonary disease
C_T	-	Cycle threshold
DAMP	-	Danger-associated molecular pattern
DTT	-	Dithiothreitol
ECM	-	Extracellular matrix
ENaC	-	Epithelial sodium channel
ERK	-	Extracellular signal regulated kinase
FAK	-	Focal adhesion kinase
FBS	-	Fetal bovine serum
FN3	-	Fibronectin type III
GM-CSF	-	Granulocyte macrophage colony-stimulating factor
GOLD	-	Global Initiative for Chronic Obstructive Lung Disease
Grb2	-	Growth factor receptor-bound protein 2
His_6-tag	-	Hexahistidine-tag
IFN-γ	-	Interferon-gamma
Ig	-	Immunoglobin
IκB	-	Inhibitory κB
IL-	-	Interleukin-
IP	-	Immunoprecipitation

IP-10	-	Interferon-gamma-induced protein-10
JNK/SAPKs	-	C-Jun N-terminal kinase/stress-activated protein kinase
LABA	-	Long acting beta agonist
LPS	-	Lipopolysaccharide
MAPK	-	Mitogen-activated kinase protein
MBP	-	Maltose binding protein
MCP-1	-	Monocyte chemotactic protein 1
MHC	-	Major histocompatibility complex
MIDAS	-	Metal-ion-dependent adhesion site
MIP-1α	-	Macrophage inflammatory protein 1α
MMP	-	Metalloproteinases
MUC5AC	-	Mucin 5AC
NF-κB	-	Nuclear factor-kappa B
NHBE	-	Normal human bronchial epithelial
NK	-	Natural killer
P38	-	P38 mitogen-activate protein kinases
PAMP	-	Pathogen-associated molecular pattern
PAS	-	Period acid-Schiff stain
PI3K	-	Phosphatidylinositol 3-kinase
Pen-strep	-	Penicillin-streptomycin
PRR	-	Pattern recognition receptor
Pyk2	-	Proline-rich tyrosine kinase-2
SEM	-	Standard error of the mean
SNP	-	Single nucleotide polymorphisms
STAT	-	Signal Transducer and Activator of Transcription
TCR	-	T-cell receptor
TEV	-	Tobacco Etch Virus
TGFβ1	-	Transforming growth factor-β1
Th	-	T helper
TLR	-	Toll-like receptor
TMEM16A	-	Transmembrane member 16A

TNF-α	-	Tumor necrosis factor-α
VEGF	-	Vascular endothelial growth factor
VWA	-	Von Willebrand factor type A
WHO	-	World Health Organization

INTRODUCTION

1.1 Rationale

Inflamed airway diseases such as asthma, cystic fibrosis, and chronic obstructive pulmonary disease affect one in seven people in North America. The hallmarks of these inflamed airway diseases include goblet cell hyperplasia, mucus hypersecretion and airway inflammation. If inflammation is not resolved, it could lead to airway remodeling, mucus hypersecretion, and tissue destruction. The consequences of these pathological conditions will lead to bacterial infection, chronic bronchitis, airflow obstruction, etc.

During airway inflammation, epithelial cells secrete high levels of pro-inflammatory cytokines and hCLCA1. The pro-inflammatory cytokines stimulate resident alveolar macrophages to release chemokines. The chemokines then recruit more macrophages to the site of inflammation and even more pro-inflammatory cytokines are released, and the inflammation is amplified. hCLCA1, a gene belong to the *CLCA* gene family, has well-documented pleiotropic effects in mucus regulation and ion channel modulation. However, the mechanism by which hCLCA1 exerts its effect remains unknown.

Since hCLCA1 proteins are secreted in the same proximity as the macrophages during airway inflammation, we hypothesize that hCLCA1 also has the ability to regulate the immune response in macrophages. Macrophages are one of the most important immune cells in humans. They have multiple functions including initiation of inflammatory response, resolution of inflammation, and tissue repair. Interaction of hCLCA1 and macrophages might explain how hCLCA1 is responsible for many different physiological functions.

1.2 Objectives

1. To determine if secreted hCLCA1 could act as a signaling molecule to induce cytokine expression in macrophages

2. To determine the functional domain of hCLCA1 and the signal transduction pathway that is involved in macrophage activation

1.3 Hypotheses

1. Secreted hCLCA1 activates and induces pro-inflammatory cytokine expression in macrophages

2. Von Willebrand Factor Type A (VWA) domain within hCLCA1 is responsible for up-regulating cytokine expression in macrophages

3. Mitogen-activated protein kinase (MAPK) or nuclear factor-kappa B (NF-κB) signaling pathways are involved with hCLCA1's VWA domain stimulation

LITERATURE REVIEW

2.1 Calcium-activated chloride channel (CLCA) proteins

2.1.1 *Backgrounds*

The *CLCA* genes (*CL* stands for chloride-channel modulating and *CA* for calcium-activated) were initially identified as the pore subunits of a calcium-activated chloride channel[1]. They were subsequently renamed to be chloride channel accessory proteins due to accumulating experimental data and advancement in bioinformatics. Of all the CLCA genes, human hCLCA1 and its murine ortholog mCLCA3 have been the most extensively studied. The gene products have pleiotropic effects, generating secreted and membrane-associated proteins that increase mucus production, airway responsiveness, and increase calcium-activated chloride channel conductance. They are also highly expressed in the airway epithelium in inflammatory airway diseases such as cystic fibrosis, asthma, and chronic obstructive pulmonary disease. However, it is still unclear how hCLCA1 or mCLCA3 can exert such pleiotropic effects.

2.1.1.1 *Initial model of CLCA proteins*

The initial member of the CLCA family was identified through immuno-precipitation using an antibody against a 38 kDa protein (anti-P38) in bovine tracheal epithelium in 1992[2]. The proteins immuno-purified using the antibody yielded products of varying molecular weights, 38, 62-64 and 140 kDa. The author concluded that the varying molecular weight proteins were monomeric, dimeric and tetrameric forms of the same product. It was suggested that the 140 kDa protein constituted the functional channel by linking four identical 38 kDa subunits through disulfide bonds. When reconstituted into lipid bilayers, the protein gave rise to anion conductance with an $I^- > Cl^-$ selectivity, and it was sensitive to the chloride channel blocker DIDS. Consistent with the channel being linked by disulfide-bonds, the channel activity was abolished when it was treated with dithiothreitol (DTT). Similarly, a protein involved in conductive chloride transport was also discovered in porcine ileal brush-border membrane in 1991[3], this protein was later cloned and identified as pCLCA1 in 2000[4].

The first clone of hCLCA1 was being reported in 1995. By using the anti-P38 antibody mentioned above, Cunningham et al. cloned a cDNA thought to encode the putative chloride channel expressed in epithelial cells in bovine trachea[1]. The cloned cDNA encoded for a 903 amino acid long protein, and it was believed to be a Ca^{2+}/calmodulin-dependent protein kinase-regulated chloride channel. This protein was predicted to have a membrane protein structure with at least four transmembrane-spanning regions. When expressed in Xenopus oocytes, the protein displayed outwardly rectified current that was anion-selective. The current was increased following exposure to calcium ionophore and inhibited by channel inhibitors. The authors concluded that the protein encoded a calcium-activated chloride channel (CaCC) and the current was similar to the electrophysiological characteristics of previous detected CaCC. This protein was subsequently named bCLCA1.

Over the next few years, several members of this gene family were cloned and identified from different mammalian species using gene screening (human, mouse, bovine)[4-12], and they were found to function as CaCCs as well. This was supported by electrophysiological experiments in which heterologous expression of CLCA isoforms from various species (human, mouse, bovine, pig) in HEK293 or NIH/3T3 cells increased chloride currents in response to increasing calcium in the cytosol[13-15].

2.1.1.2 *Current and evolving model of CLCA proteins*

However, there were inconsistencies to the initial model that had suggested CLCA proteins were CaCCs themselves. Structurally, the predicted transmembrane-spanning regions for some of the CLCAs included a soluble von Willebrand factor type A (VWA) domain, which was inconsistent with being a channel[6]. Typically, amino acids in the transmembrane-spanning regions of an ion channel are organized into alpha-helix or beta-barrel structure. Functionally, members of the CLCA family were linked to roles other than ion channels, playing a role in regulation of mucus production (mCLCA3) and tumor suppression (CLCA2)[16, 17]. Moreover, some of the CLCA proteins were reported to be cell adhesion molecules (e.g. hCLCA2) or to be truncated with no transmembrane-spanning regions (hCLCA3), thus they could not form the chloride channels themselves[18].

The accumulating experimental results and advancement in bioinformatics led to a new model for CLCA structures and functions. The current model described CLCA proteins as soluble secreted molecules with the exception of a subset of CLCA proteins that contain a C-terminal membrane anchoring region[19, 20]. A common feature shared by the CLCA family of proteins is the presence of a proteolytic cleavage site near the C-terminus, and all the CLCA isoforms tested in mammalian cell culture system seemed to be processed similarly[5, 19-26]. Generally, a precursor ~120 kDa glycoprotein is cleaved into two products: a ~85 kDa N-terminal subunit and a ~35 kDa C-terminal subunit. However, the size of the proteins seems to be cell-line- and tissue-dependent[17]. All N-terminal subunits are secreted into the extracellular space while the C-terminal subunits are either secreted or retained inside the cells depending on the CLCA ortholog[20-23]. Today, CLCA genes have been renamed to chloride channel accessory proteins. Although CLCA family proteins do not form the pore subunits of the CaCCs, they remain important to many physiological functions including regulation of chloride channel conductance, modulation of mucus expression, regulation of immune responses, etc (Table 2.1). To date, CLCA genes from other mammalian species have been identified, and this has allowed the categorization of CLCA from different species into different subgroups based on the level of sequence similarity (Table 2.2, Figure 2.1 and 2.2).

Table 2.1. Tissue expressions and functions of mammalian CLCA gene homologs.

Gene	Tissue Distribution	Functions
hCLCA1	Airway mucous cells, intestinal goblet cells, uterus, stomach, testis, kidney	Increases CaCC conductance, induces goblet cell metaplasia, regulates airway inflammation
hCLCA2	Lung, trachea, mammary gland, testis, prostate, bladder, stomach	Increases CaCC conductance, tumor promoter and suppressor
hCLCA3	Lung, trachea, mammary gland, thymus, spleen	No function reported
hCLCA4	Colon, bladder, uterus, trachea, stomach, prostate, mammary gland, brain	Possible disease modifier for cystic fibrosis
mCLCA1	Lung, aorta, spleen, bone marrow, lymph nodes, brain, kidney, skin, liver, spleen, intestine, cecum, dorsal root ganglion, breast	Increases CaCC conductance, tumor promoter and suppressor
mCLCA2	Involuted mammary tissues, thymus, colon, intestine, bladder, epididymis, vesicular gland, skin, dorsal root ganglion	Tumor suppressor, possible apoptotic inducer
mCLCA3	Mucous cells of small intestines, colon, lung, trachea, uterus, stomach	Induces goblet cell metaplasia, possible inducer for airway hyper-responsiveness, induces airway inflammation
mCLCA4	Smooth muscle of lung, heart, uterus, bladder, stomach, aorta, skeletal muscle, intestinal goblet cells	Increases CaCC conductance that mimics native chloride conductance in smooth muscle
mCLCA5	Eye, spleen, heart, intestine, lung, skeletal muscle, stomach, testis, dorsal root ganglion	Increases CaCC conductance, induces goblet cell metaplasia, tumor suppressor
mCLCA6	Intestine, stomach	Increases CaCC conductance, potential regulator for CFTR
mCLCA7	No expression reported	No function reported
mCLCA8	No expression reported	No function reported
pCLCA1	Surface epithelium in ileum, surface epithelium and submucosal glands in trachea, sublingual, submandibular and parotid salivary glands	Increases CaCC conductance, increases cAMP-dependent chloride conductance
pCLCA2	Skin	Potential role in structural integrity of the skin
pCLCA4	Lung	No function reported

Table 2.2. Human CLCAs and their animal homologs. h = human, m = murine, p = porcine, b = bovine, e = equine.

Human CLCAs	hCLCA1	hCLCA2	hCLCA3	hCLCA4
Animal homologs	mCLCA3 pCLCA1 bCLCA1 eCLCA1	mCLCA5 pCLCA2 bCLCA2 eCLCA2	mCLCA1 mCLCA2 mCLCA4 bCLCA3	mCLCA6 mCLCA7 mCLCA8 pCLCA4 bCLCA4 eCLCA4

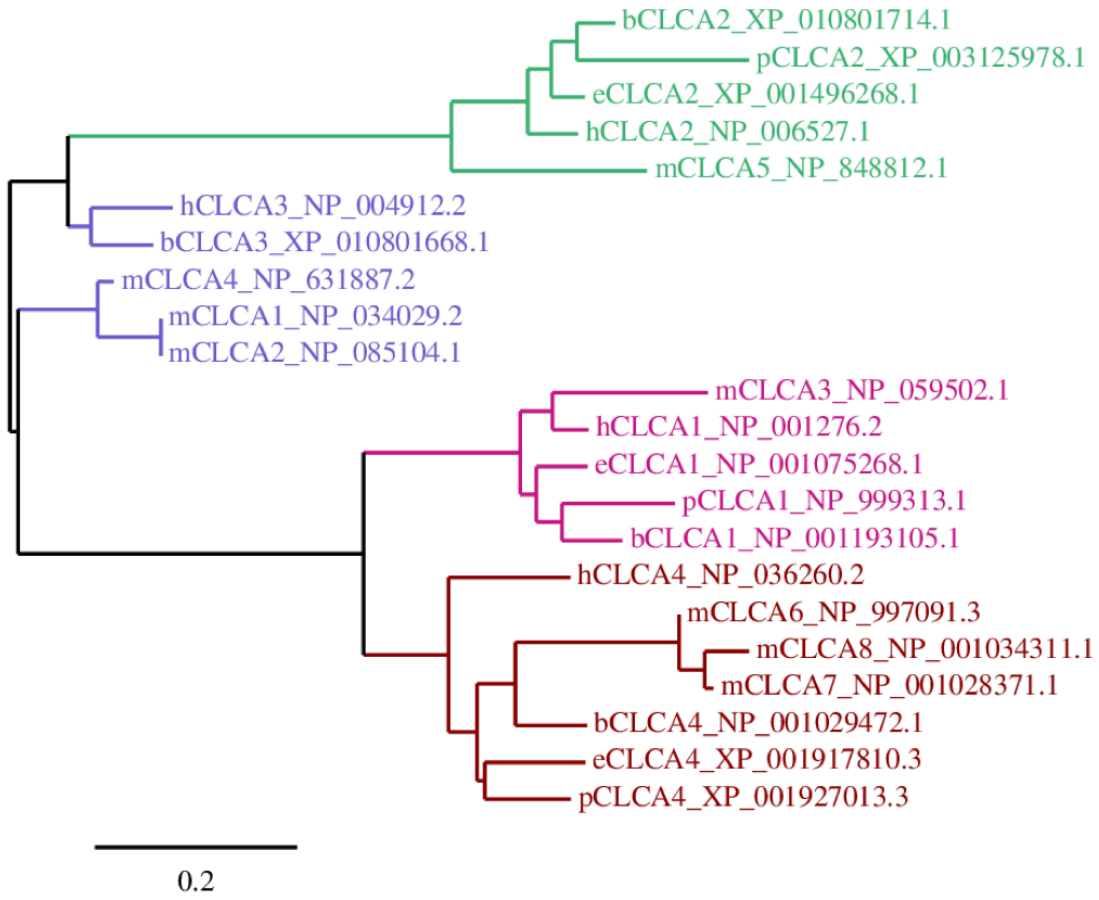


Figure 2.1. Phylogenetic tree for CLCA family members in human, mouse, pig, horse, and cow. The analysis was performed on the *Phylogeny.fr platform*. Sequences were aligned with MUSCLE (v3.8.31), the phylogenetic tree was reconstructed using PhyML program (v3.1/3.0 aLRT), and the graphical representation was generated with TreeDyn (v198.3)[27-33]. The bar represents 20% sequence diversity. Each color represents a homologous group comparing to each human CLCA gene.

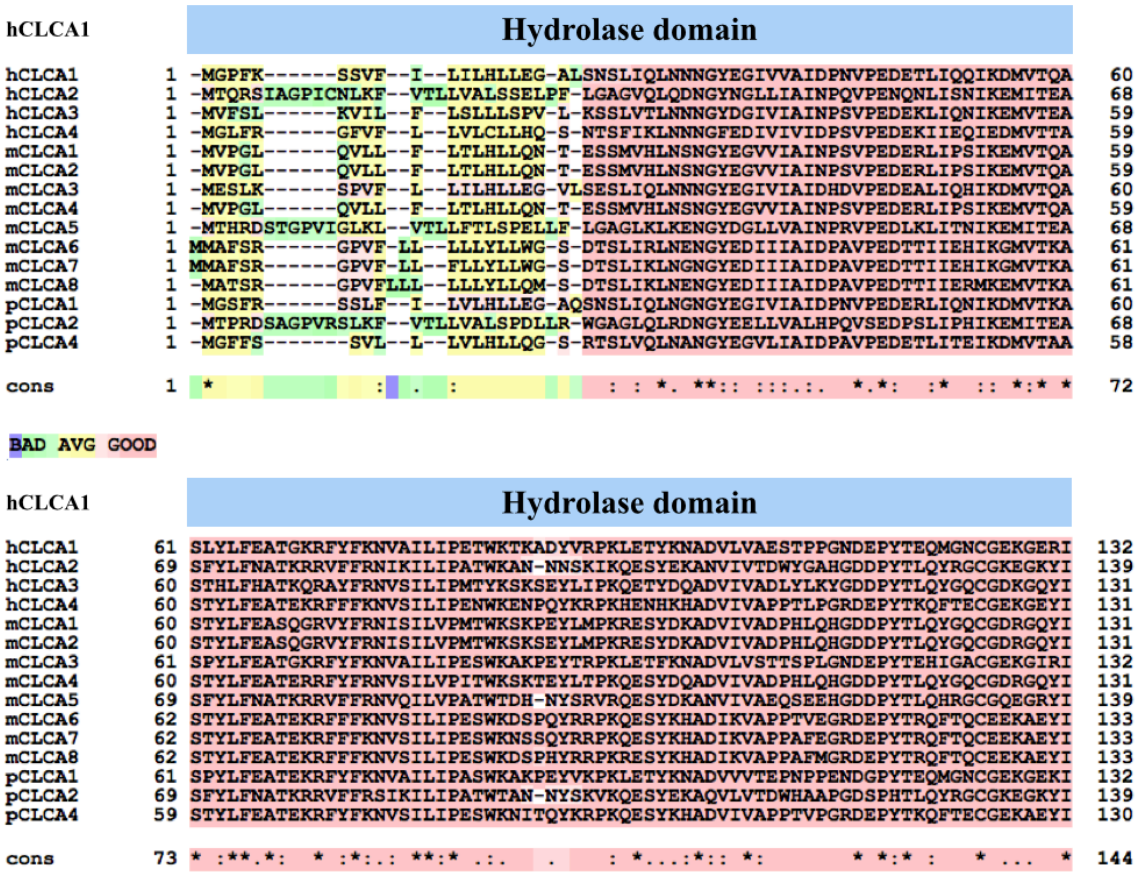
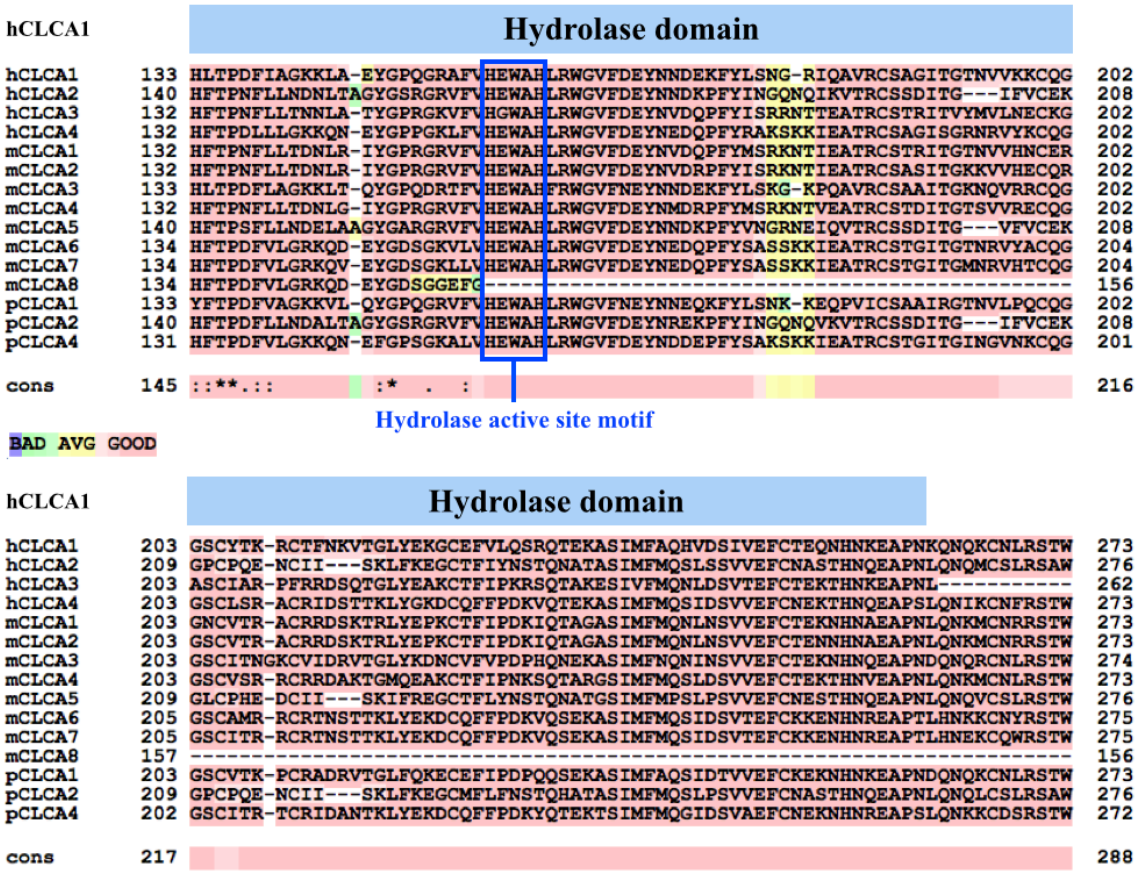


Figure 2.2. Annotated sequence alignment for CLCA proteins in human, mouse, and pig. Sequence analysis and alignment were performed with T-Coffee multiple sequence alignment program. The color indicates the levels of similarity between different CLCA homologs, with purple being the least similar to pink being the most similar. The domains (hydrolase domain, VWA domain, and FN3 domain) are identified based on hCLCA1 sequence. The blue arrows indicate the sites of metal-ion-dependent adhesion site (MIDAS) motifs. The blue boxes indicate the location of hydrolase active site motif or β4-intergrin motif. Sequence conversation is denoted by consensus symbols: an asterisk (*) indicates positions which have a single, fully conserved residue, a colon (:) indicates conservation between groups of strongly similar properties, and period (.) indicates conservation between groups of weakly similar properties.



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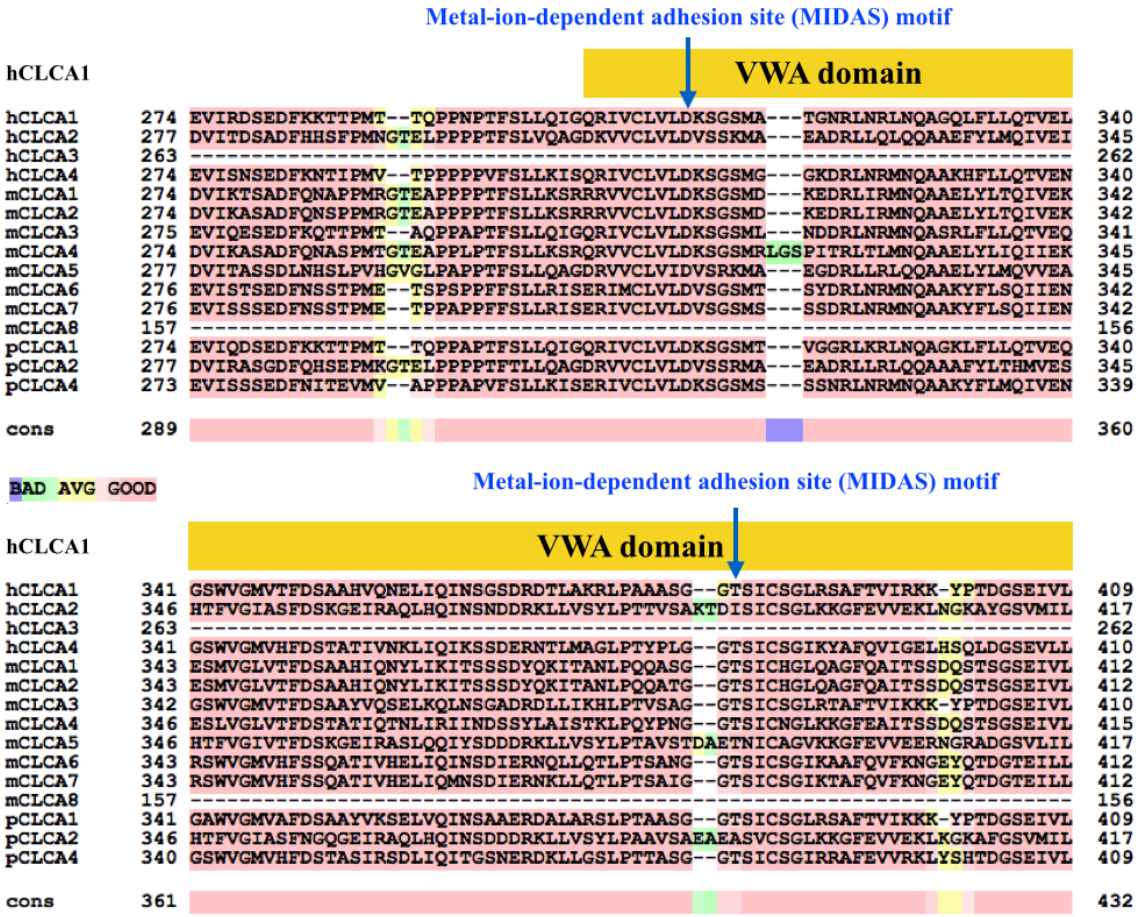

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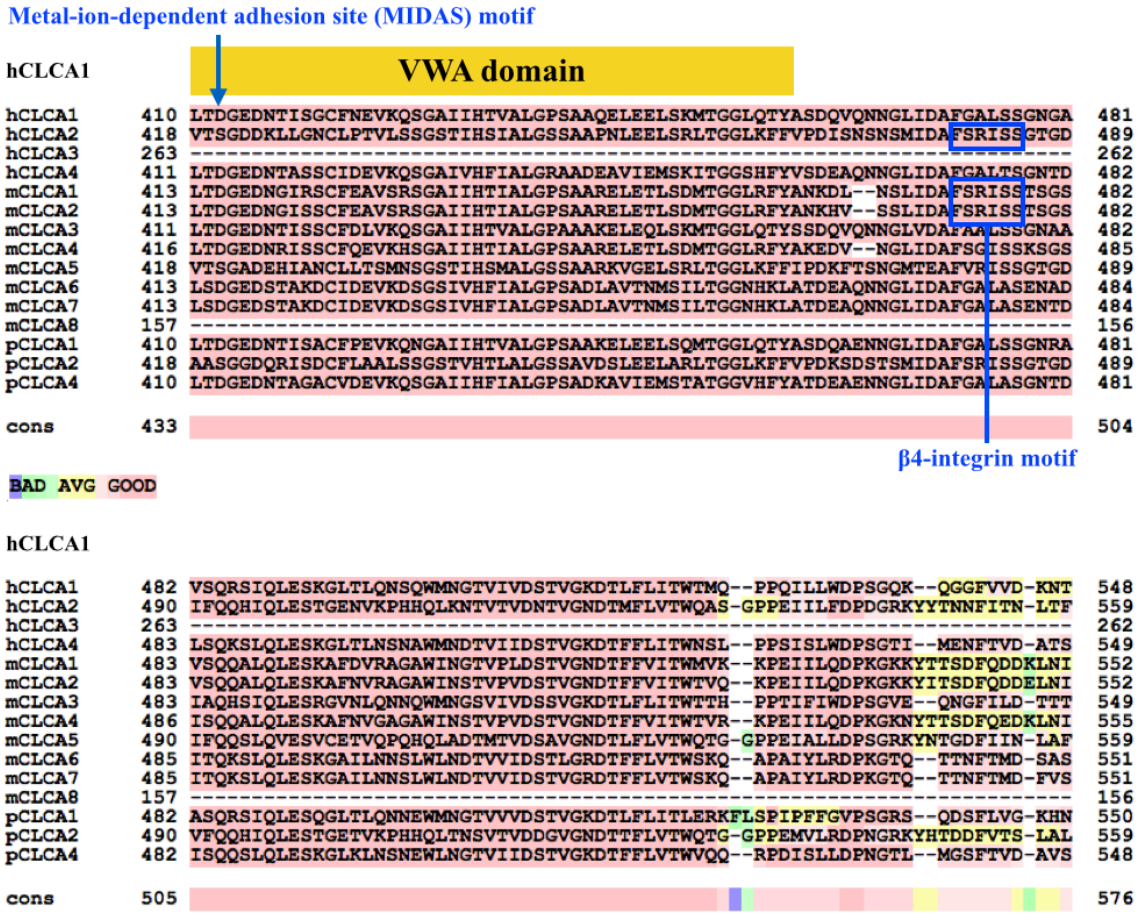

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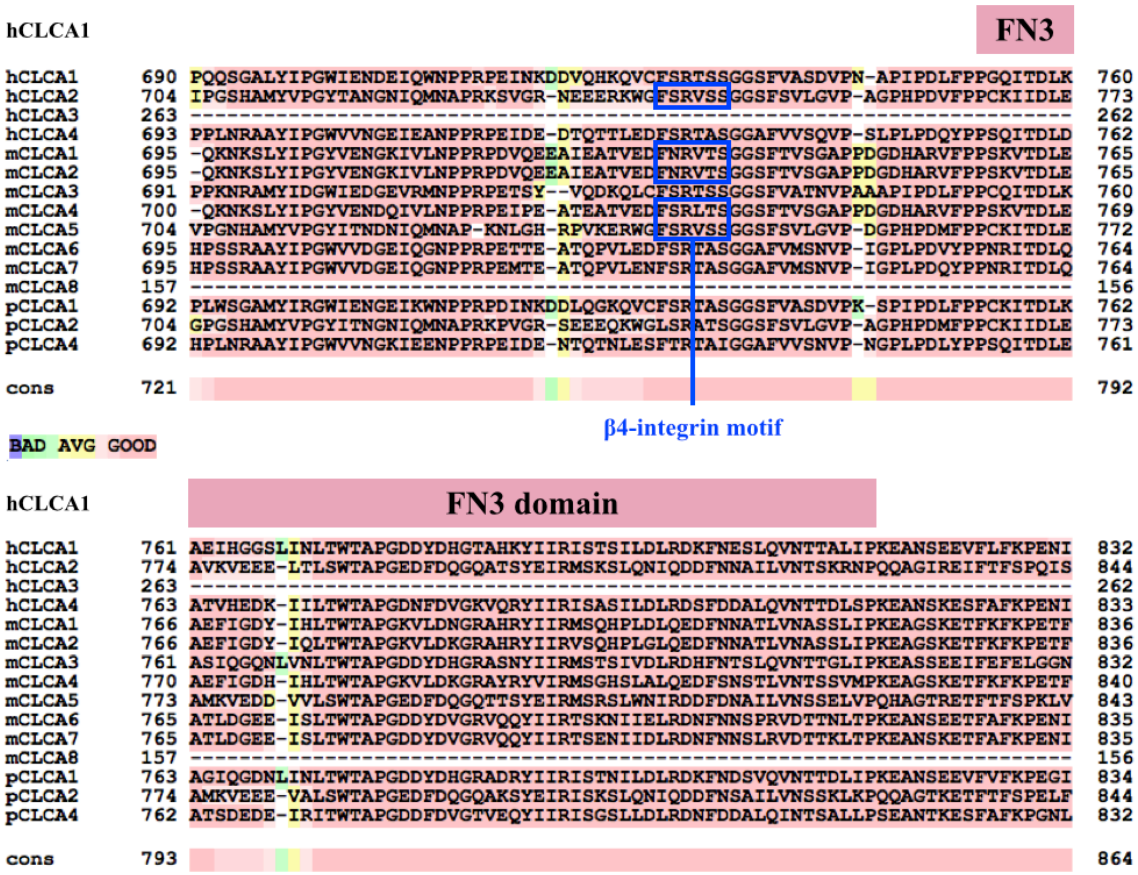

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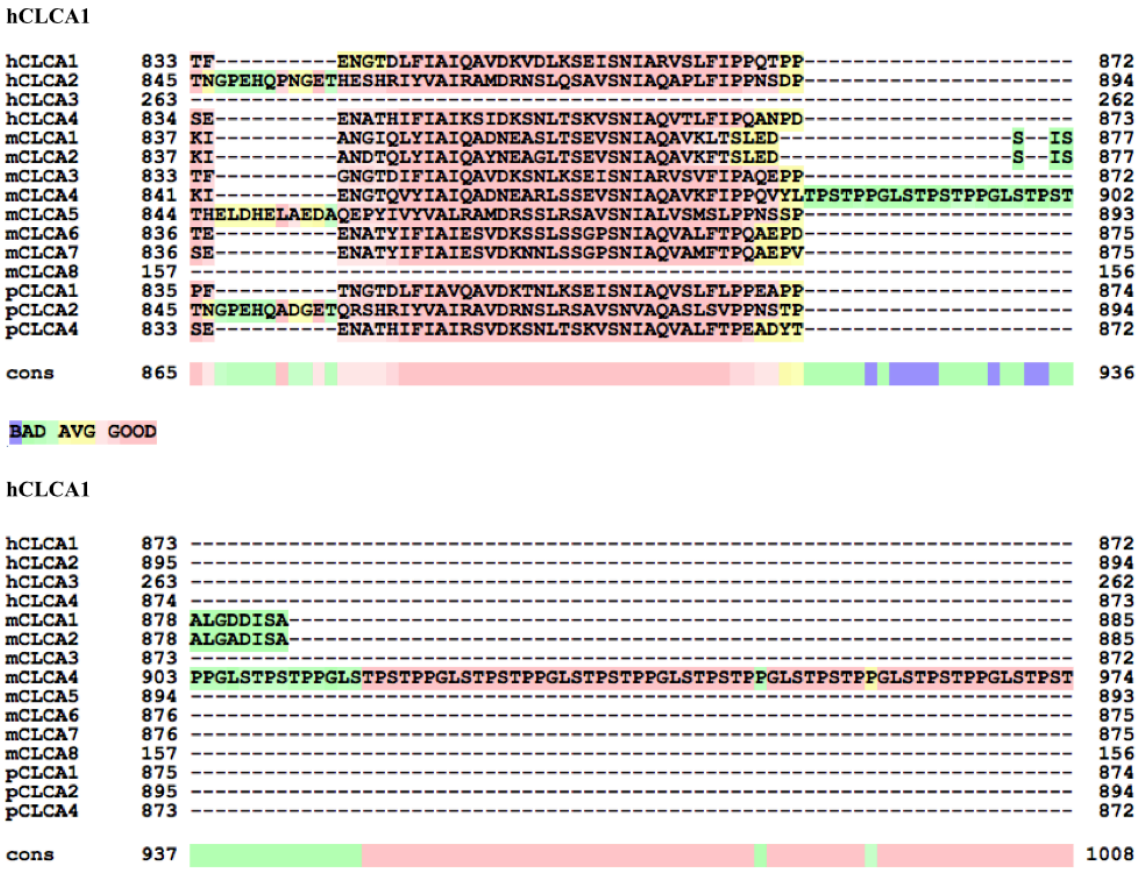

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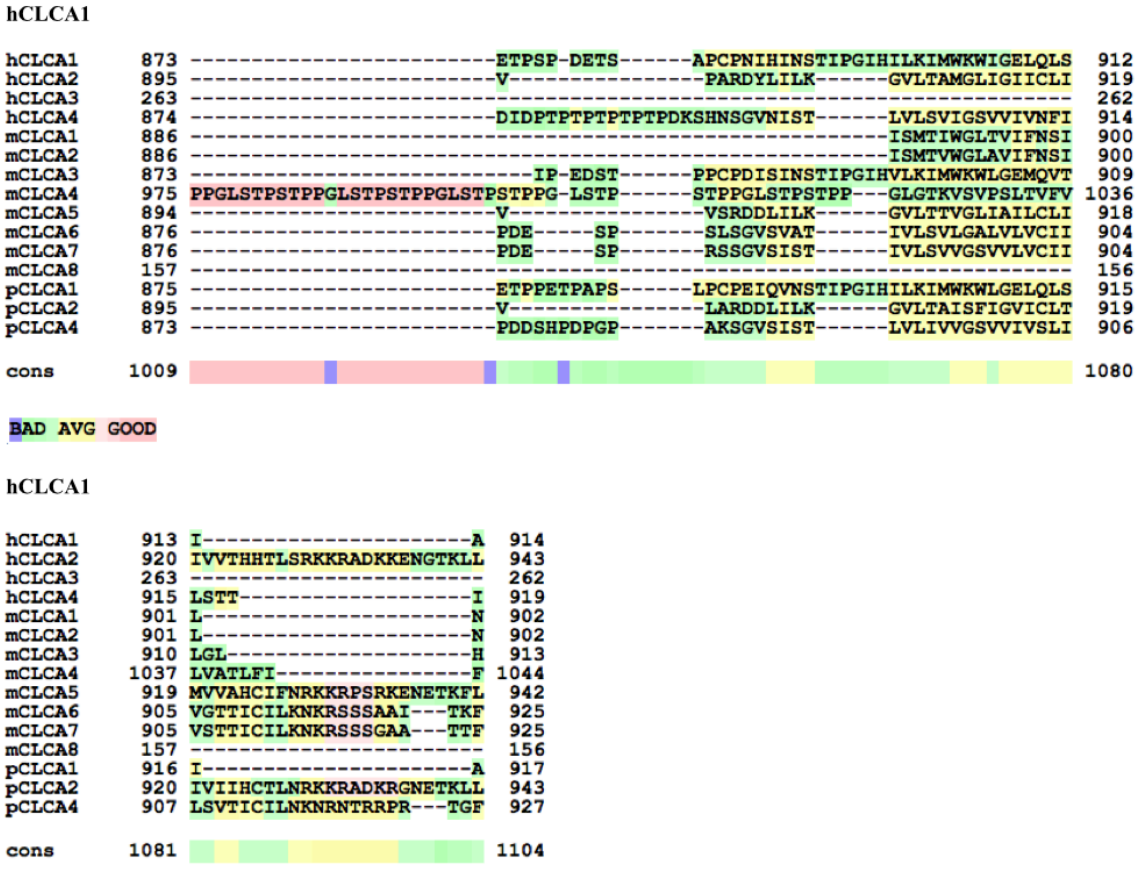


Figure 2.2. Continued

2.1.1.3 *CLCA homologs in mammalian species*

To date, CLCA homologs have been identified in many species (human, mouse, pig, horse, etc.). This section will cover the tissue distributions as well as the functions of different CLCAs expressed in human, mouse, and pig. There are 4 identified CLCAs in human, 6 or more in mouse, and 3 in pig.

2.1.1.3.1 *Human CLCAs*

The first human CLCA gene identified was hCLCA1 and it is the most extensively studied human CLCA gene[6]. It is expressed in multiple tissues, predominantly in the mucus-secreting cells in the airways and small and large intestines[5, 34], with much lower expression levels in the uterus, stomach, testis, and kidney[35]. Transient expression of hCLCA1 in HEK293 cell line increased calcium-activated chloride channel (CaCC) conductance[6, 36]. Recently, a study demonstrated hCLCA1's ability to increase CaCC conductance by increasing the surface expression of endogenous transmembrane member 16A (TMEM16A), the actual calcium-activated chloride channel[37]. hCLCA1 has also been intensely studied with its association with mucus production. Experimental results demonstrated that transient expression of hCLCA1 *in vitro* could induce mucus production[38-40], and hCLCA1 was reported to be significantly induced in the airway epithelia of patients suffering from airway diseases such as cystic fibrosis (CF), asthma, and chronic obstructive pulmonary disease (COPD) (See section 2.2; Respiratory diseases)[34, 41-44]. To add another layer of complexity to the functions of hCLCA1, we were the first ones to demonstrate that secreted hCLCA1 could act as a signaling molecule and induce pro-inflammatory cytokine expression in macrophages, suggesting its role in immune regulation[45]. We were also the first to show that the secreted form of hCLCA1 possesses physiological functions. This signaling property of hCLCA1 might explain how hCLCA1 can have such a pleiotropic effect toward different physiological functions.

The second member of the hCLCA family, hCLCA2, is expressed in the lung, trachea and mammary gland[12], with weak expression in testis, prostate, bladder and stomach[35]. Similar to hCLCA1, transient expression of hCLCA2 in HEK293 also increased CaCC conductance[12].

Interestingly, hCLCA2 seems to play a role in both tumor metastasis and suppression. One study demonstrated that the β4-binding motif in the VWA domain of hCLCA2 could bind to the β4 integrin expressed on breast cancer cells, and this led to metastatic spread of the cancer[16]. However, β4-integrin motif is only present in hCLCA2, mCLCA1, and mCLCA2 (Figure 2.2). Contrary to the tumor metastatic role, hCLCA2 also seems to partake a role in tumor suppression. Gruber and Pauli found that hCLCA2, which expressed abundantly in normal breast epithelial cells, was depleted in transformed tumorigenic cell lines[18]. In addition, when the highly metastatic breast cancer cell line transgenically expressing hCLCA2 was injected into nude mice, it decreased the capacity of these cells for invasion and tumor generation[18].

Unlike other CLCA homologs, hCLCA3 is a truncated CLCA protein with a premature stop codon, and the expressed protein is secreted into the medium *in vitro*[9]. It was reported to express in the lung, trachea, mammary gland, thymus and spleen[9]. However, no function has been reported for hCLCA3. The fourth member of human CLCA, hCLCA4, is expressed in various tissues including the colon, bladder, uterus, trachea, stomach, prostate, mammary gland, and it is the only human CLCA that expresses in the brain[35]. However, the function of hCLCA4 remains unknown, although it was suggested to be a potential modifier of disease severity in cystic fibrosis[46].

2.1.1.3.2 *Mouse CLCAs*

mCLCA1 was the first identified murine CLCA gene, and it was found to express in a variety of tissues including the lung, aorta, spleen, bone marrow, lymph nodes, brain, kidney, skin, liver, spleen, intestine, cecum, brain, dorsal root ganglion and breast[11, 18, 47, 48]. Similar to other CLCA orthologs, expression of mCLCA1 in HEK293 or Xenopus oocytes increased CaCC currents[5, 11]. Like hCLCA2, mCLCA1 seems to partake in both tumor promotion and suppression. One study showed that the binding of mCLCA1 and β4-intergrin expressed on mouse skin melanoma cell line activated focal adhesion kinase (FAK), which activated downstream extracellular signal regulated kinase (ERK) and promoted proliferation of tumor cells (see section 2.3.3.2; MAPKs)[49]. In addition, this study found that the FAK-mediated signaling to ERK is Src-dependent. The mCLCA1-β4-intergrin interaction promoted FAK autophosphorylation at

Tyr397, which created a binding site for Src. Interaction with Src further phosphorylated FAK, creating an SH2-binding site for growth factor receptor-bound protein 2 (Grb2), which subsequently activated the downstream signaling to ERK. It is important to note that Grb2 is also an intermediate adaptor protein that can activate downstream p38 mitogen-activated protein kinases (p38). However, the author did not investigate p38 activation likely because ERK is involved in cell proliferation while p38 is involved in inflammation. This study looked at three different signaling targets that were immediately downstream of focal adhesions: focal adhesion kinase (FAK), proline-rich tyrosine kinase-2 (Pyk2), and phosphatidylinositol 3-kinase (PI3K). However, only FAK was strongly activated upon mCLCA1 interaction. It is important to note that activation of PI3K subsequently phosphorylates serine/threonine kinase Akt, and phosphorylated Akt phosphorylates IKKα, resulting in activation of nuclear factor-kappa B (NF-κB) pathway (See Section 2.3.3.1; Nuclear factor-kappa B)[50]. Although mCLCA1-β4-integrin interaction did not promote PI3K activation, it does not exclude the possibility that the β4-binding motif in other CLCAs could activate PI3K pathway that will eventually lead to NF-κB activation. Contrary to the above study, another study reported that mCLCA1 was down-regulated in human breast cancer cell line[51]. When mCLCA1 was reintroduced into the tumor cell line by transfection, reduced growth rate and survival rate was observed[51]. These contrary results suggest that the effect of mCLCA1 on tumor cell growth might be cell-line or species-dependent.

The second member of the murine CLCA family, mCLCA2, is an ortholog of mCLCA1 that expresses in low levels in most of the tissues[48]. Strong expression of mCLCA2 was observed in involuted mammary tissues, but not in pregnant or lactating tissues[48, 52]. With the highest expression found in cells with high turnover, mCLCA2 was suggested to have an apoptotic role in these tissues. Indeed, further experiment indicated that mCLCA2 expression was induced in response to apoptotic stimuli[51]. When mCLCA2 was transfected into tumor cell line, it elicited a survival inhibitory effect which was more potent than that induced by mCLCA1[51].

mCLCA3, also known as gob-5, is a murine ortholog of hCLCA1 and is the most well-studied CLCA gene in mouse. It expresses in the mucous cells of different tissues including small intestine, colon, lung, trachea, uterus and stomach, with the highest expression in gastrointestinal and respiratory tracts[10, 17]. Although expression of mCLCA3 *in vitro* generated CaCC

conductance, the effect cannot be reproduced *in vivo* with mCLCA3 knockout mice[53]. This could be attributed to the redundancy of CLCAs in mice, in which other CLCAs compensate for the loss of mCLCA3. However, most of the attention of mCLCA3 was focused in its association with mucus production. Similar to its human ortholog hCLCA1, transient expression of mCLCA3 *in vitro* caused significant increase in mucus production[43]. It was reported that mCLCA3 expression was selectively induced in asthmatic mice, and administration of adenovirus-expressing antisense mCLCA3 inhibited the development of goblet cell metaplasia and airway hyper-responsiveness while overexpression of mCLCA3 exacerbated the asthma phenotype[43]. This was partly consistent with a study that demonstrated mCLCA3 gene transfer induced goblet cell metaplasia but not airway hyper-responsiveness[54]. These results were supported by a recent study that intranasal administration of mCLCA3 antibody significantly reduced airway inflammation, goblet cell hyperplasia and mCLCA3 expression in asthmatic mice[55].

The fourth member of the murine CLCA, mCLCA4, is expressed in smooth muscle of uterus, lung, heart, bladder, stomach, aorta, skeletal muscle and in the mucous cell in gastrointestinal tracts[26]. Expression of mCLCA4 in HEK293 exhibited CaCC conductance that mimicked the native calcium-activated chloride channels in smooth muscles[26]. However, no other physiological functions of mCLCA4 have been reported. One study showed that the luminal sorting motifs on mCLCA4 sequence was required for export from endoplasmic reticulum and subsequent proteolytic processing. The same study demonstrated that mutation in the luminal sorting motifs cause mCLCA4 to be trapped in the endoplasmic reticulum and have impaired proteolytic cleavage of itself[22]. It is likely that other CLCAs are processed in a similar manner since most CLCAs have high sequence homologies at their N-terminal (Figure 2.2).

mCLCA5 is a murine ortholog of hCLCA2. Similar to hCLCA2, mCLCA5 is expressed in a variety of tissues including heart, intestine, lung, skeletal muscle, stomach, testis, dorsal root ganglion, and highest expression in spleen and eye[47, 56]. Like other CLCAs, transfection of mCLCA5 in HEK293 also generated CaCC current[56]. The expression of mCLCA5 was found to increase in mCLCA3 knockout mice experiencing goblet cell metaplasia. In addition, the author showed mCLCA5 gene transfer was sufficient to drive goblet cell metaplasia in mice[54], suggesting mCLCA5 might compensate for the loss of mCLCA3 in mice. Similar to its human

ortholog hCLCA2, mCLCA5 expression was found to decrease in tumor cells while increase under serum-free conditions, suggesting its role as an apoptotic inducer. This was supported by the arrest of cell growth and colony formation after mCLCA5 was expressed in the tumor cell line[24].

mCLCA6, which is a murine ortholog of hCLCA4, expresses predominantly in intestine and stomach[56]. Expression of mCLCA6 also generated CaCC conductance[56]. One study reported that mCLCA6 colocalized with cystic fibrosis transmembrane regulator (CFTR) at the apical surface of colonic crypt cells. This suggested a direct or indirect role of mCLCA6 in transepithelial anion conductance in the mouse intestine[19]. Using BLAST program to search for homologs of the mCLCA3, Patel et al. discovered mCLCA7 and mCLCA8 gene. However, no expression and functional studies have been reported[57].

2.1.1.3.3 *Pig CLCAs*

The first member of porcine CLCA family, pCLCA1, is an ortholog of hCLCA1 and mCLCA3. It expresses predominantly in the surface epithelium in ileum, surface epithelium and the underlying submucosal glands in the trachea, and low levels in sublingual, submandibular and parotid salivary glands[4, 58]. pCLCA1 was suggested to be a mediator for both CaCC and cyclic adenosine monophosphate (cAMP)-dependent chloride channel[4, 59, 60]. Increased calcium-activated chloride conductance was observed when expressed in NIH/3T3 cells[4]. In addition, expression of pCLCA1 in an intestinal epithelial CaCO-2 cell line also induced cAMP-dependent chloride conductance, possibly through the regulation of CFTR[59]. The same group subsequently showed that the regulation of cAMP-dependent chloride channel by pCLCA1 persisted in mature polarized CaCO-2 cells, in which the calcium-activated chloride conductance disappeared[60].

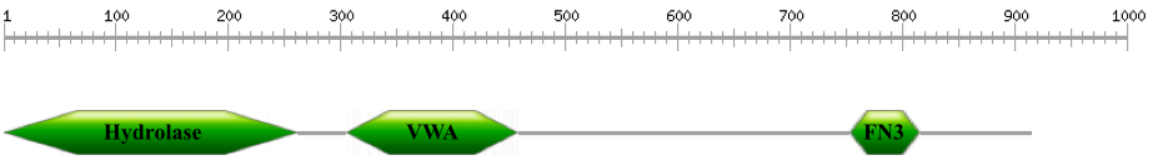
The second member of the porcine CLCA family, pCLCA2, is an ortholog of human hCLCA2 and murine mCLCA5 and is the only member of CLCA that expresses in the skin, and the author suggested that pCLCA2 might play a role in the structural integrity of the skin[61].

The third member of the porcine CLCA gene, pCLCA4, is an ortholog of hCLCA4 and mCLCA6 that expresses strongly in the lung[62]. However, similar to pCLCA2, no functional studies have been conducted on these genes.

2.1.2 *Protein structure of hCLCA1*

2.1.2.1 *Molecular characteristics and functional domains*

The *hCLCA1* gene is located on chromosome 1 (1p22.3) and encodes a 914 amino acid protein with a calculated molecular mass of 100.3 kDa. Using the ScanProsite bioinformatics tool, it is predicted that hCLCA1 contains 18 potential sites for N-myristoylation, 13 consensus sites for protein kinase C phosphorylation, 2 consensus sites for amidation, 12 consensus sites for casein kinase II phosphorylation, 3 consensus sites for Ca^{2+}/calmodulin dependent kinase II phosphorylation, and 8 potential sites for asparagine-linked glycosylation[14, 63]. Post-translational modifications such as myristoylation and glycosylation could be vital to hCLCA1 signal transduction ability. Myristoylation allows weak protein-protein and protein-lipid interaction, and it has been shown to play an essential role in protein-protein interaction and signal transduction[64]. On the other hand, glycosylation is known to modulate the structure and function of signaling molecules[65-67]. Proteomic bioinformatics has indicated that hCLCA1 contains functional domains that are commonly shared among CLCA gene family[5, 19-26]. These specifically include domains such as hydrolase domain, von Willebrand Factor type A domain, and Fibronectin type III (FN3) domain (Figure 2.3).



Hydrolase = Hydrolase domain
VWA = von Willebrand factor type A domain
FN3 = Fibronectin type III domain

Figure 2.3. Structural scheme of hCLCA1 protein. Using NCBI's conserved domains database, three domains were predicted in hCLCA1. The hydrolase domain lies between 1-261 residues at the N-terminal of the protein. The von Willebrand factor type A (VWA) domain is located between 305-458 residues, and the fibronectin type III (FN3) domain is located between 753-816 residues of hCLCA1.

2.1.2.1.1 *Hydrolase domain*

Recent studies have identified the presence of a hydrolase domain in the N-terminal region of the hCLCA1 protein (1-261 residues)[68]. The study reported that the structural prediction of the hydrolase domain was similar to that of the zinc-dependent metalloproteases with a His-Glu-x-x-His (HExxH) active site motif, and the hydrolase function was abolished when glutamate residue 157 was mutated into glutamine (HEWAH → HQWAH). A follow up study by the same group demonstrated that this HExxH motif is well-conserved in the CLCA family[69].

When hCLCA1 is expressed, it is subjected to glycosylation and possible myristoylation, increasing its molecular mass from ~100 kDa to ~130 kDa. Then the hydrolase domain autocleaves the precursor hCLCA1 into a ~90 kDa amino-terminal and ~40 kDa carboxyl-terminal subunits. Although it was reported that both subunits were secreted into the extracellular compartment[6], our results showed otherwise. From our western blot analysis, we found that only the glycosylated forms of hCLCA1 were secreted into the extracellular compartment. Both the precursor hCLCA1 and N-terminal product were secreted while the C-terminal product was retained inside the cell (Figure 2.4). Similar to previous studies, we also observed an E157Q mutation in hCLCA1 abolished the proteolytic activity. A recent study reported that the proteolytic activity of hCLCA1 was required to regulate calcium-activated chloride channel activity. This study demonstrated that hydrolase-inactive hCLCA1 prevented activation of calcium-activated chloride channel-mediated chloride transport[70]. The author proposed that the C-terminal region masks the N-terminal region in the full-length protein, and proteolytic cleavage of the full-length protein is required to expose the N-terminal region to interact with the calcium-activated chloride channel. However, our data demonstrated that hydrolase domain activity was not necessary for hCLCA1 to activate airway macrophages[45].

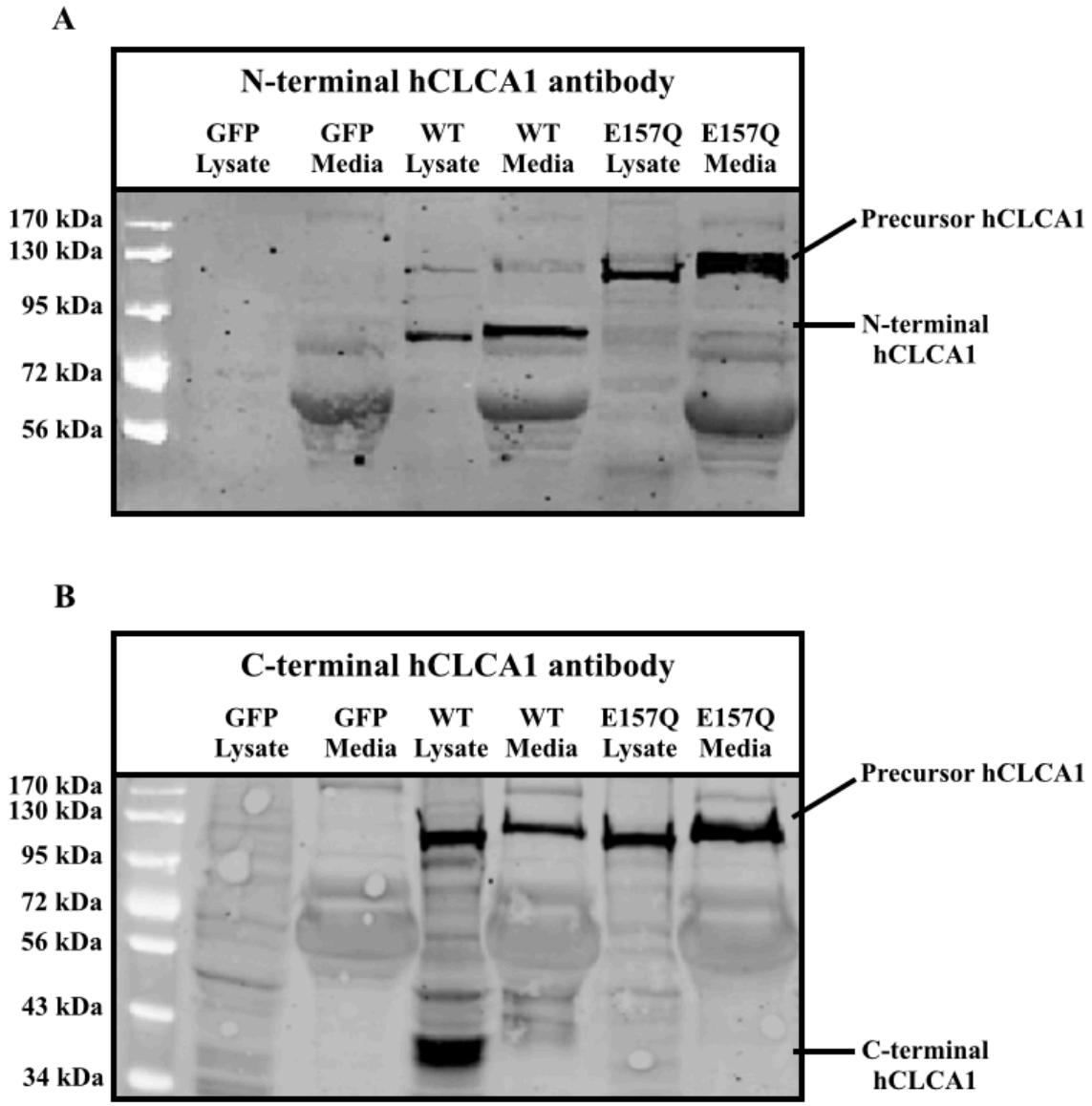


Figure 2.4. Biosynthesis, glycosylation, and secretion pattern of hCLCA1. Western blot analysis of hCLCA1-transfected HEK293 cell lysates and media using **(A)** N-terminal hCLCA1 antibody and **(B)** C-terminal antibody. All precursor, N-terminal, and C-terminal hCLCA1 products were subjected to glycosylation. Both precursor and N-terminal hCLCA1 were secreted into the extracellular compartment. In addition, higher proportion of N-terminal hCLCA1 product compared to the precursor was observed. Figure A was modified from the publication: Ching, J. C., Lobanova, L. & Loewen, M. E. (2013). Secreted hCLCA1 is a signaling molecule that activates airway macrophages. *PLoS One* **8**, e83130. Figure B was from our unpublished data.

2.1.2.1.2 *Von Willebrand factor type A domain*

A von Willebrand factor type A (VWA) domain is predicted between residues 305-458 of hCLCA1. Using NCBI's conserved domain database, hCLCA1's VWA domain is predicted to contain three metal-ion-dependent adhesion site (MIDAS) motifs.
It is a well-studied domain involved in cell adhesion, in extracellular matrix proteins, and in integrin receptors[71-73]. Studies have also demonstrated that VWA domain was also involved in regulating ion channel function. Precedents exist, as the α2δ subunit of the voltage gated calcium channel modulates its function by binding to an extracellular region of the channel pore subunit via its VWA domain[74]. Moreover, mutation studies reported that the MIDAS motif in the α2δ subunit was essential for its function in channel trafficking and modulation of calcium channel[74-77].

Our unpublished data also demonstrated that the VWA domain of hCLCA1 was responsible for macrophage activation (see section 4; Study 2). The MIDAS motifs in hCLCA1 VWA domain provide possible active sites that are responsible for macrophage activation. This idea is supported by a recent study showing that secreted hCLCA1 increased CaCC conductance[37], similar to how α2δ subunit of the voltage gated calcium channel modulates its function[74].

However, the VWA domain could also execute its function independent of its MIDAS motifs. As mentioned above, the β4-binding motif in the VWA domain of hCLCA2 was reported to bind to the β4 integrin expressed in breast cancer cells, leading to metastatic spread of the cancer[16]. A similar mechanism was also reported for mCLCA1, in which binding of mCLCA1 and β4 integrin activated the signaling molecule focal adhesion kinase (FAK), which activated downstream extracellular signal regulated kinase (ERK) and promoted proliferation of tumor cells[49]. hCLCA1 has a disrupted β4-binding motif, however this does not exclude the possibility that the VWA domain interacts with other proteins with a yet identified domain or binding motif.

2.1.2.1.3 *Fibronectin type III domain*

The fibronectin type III domain lies in the C-terminus of the hCLCA1 protein. It is predicted to lie between residues 753-816. Although the function of the FN3 domain in hCLCA1 remains unknown, its existence suggests hCLCA1 may act as a signaling molecule. Studies specifically on the FN3 domains reported that they interact with β1 integrin and mediate Src-dependent focal adhesion kinase (FAK) phosphorylation. This eventually leads to activation of downstream signaling to extracellular signal regulated kinase (ERK), in which activated ERK is involved in cell migration, proliferation, spreading, and inhibition of apoptosis[78, 79]. It has also been reported that unfolding of the FN3 domain in fibronectin induces expression of cytokines in lung fibroblast cells through toll-like receptor (TLR) and nuclear factor-kappa B (NF-κB) pathways[80].

2.1.3 *Physiological functions of hCLCA1*

2.1.3.1 *Modulation of calcium-activated chloride channel (TMEM16A) conductance*

When hCLCA1 was first identified, it was thought to form the pore subunit of a calcium-activated chloride channel. Transient expression of hCLCA1 in HEK293 cells increased CaCC conductance[6]. It was later confirmed that the increase in CaCC current by hCLCA1 expression was due to regulation of hCLCA1 on endogenous calcium-activated chloride channel (TMEM16A)[36]. TMEM16A was recently identified definitively as the first genuine CaCC in mammals[81-83]. It was concluded that hCLCA1 elevated the conductance of endogenous CaCC by lowering the energy barriers for ion translocation. A recent study reported that exogenous hCLCA1 increased CaCC current by stabilizing surface expression of TMEM16A[37]. This agrees with our proposed novel function of hCLCA1, in which secreted hCLCA1 can act as a signaling molecule to regulate different physiological responses[45].

2.1.3.2 *Association with mucus expression*

Most of the attention on hCLCA1 has been focused on its association with mucus production. Epithelial goblet cell hyperplasia and mucus hypersecretion are the hallmarks of patients

suffering from inflammatory airway diseases such as cystic fibrosis, chronic obstructive pulmonary disease, or asthma[43, 84]. The excessive mucus production by airway epithelium during airway inflammation disrupts normal airway physiology, and this predisposes the host to bacterial infection.

The involvement of hCLCA1 or its murine ortholog mCLCA3 with mucus production was first identified in 2001[43]. Nakanishi et al. identified mCLCA3 gene to be selectively induced in the lungs of an asthmatic mice model. Administration of adenovirus-expressing antisense mCLCA3 inhibited the development of goblet cell metaplasia and airway hyper-responsiveness while overexpression of mCLCA3 exacerbated the asthma phenotype. Nakanishi et al. also demonstrated that transient expression of hCLCA1 or mCLCA3 increased mucus production and mucin 5AC (MUC5AC) gene expression in NCI-H292 mucoepidermoid cell line[43]. In the same year, Zhou et al. demonstrated that mCLCA3 expression was mediated by T helper (Th)-2 cytokines (see section 2.3.2.2; Th2 cytokines)[85], and Th2 cytokines including Interleukin (IL-)-4, IL-9 and IL-13 have been implicated in the regulation of mucus production in airways[86-88]. Beside Th2 cytokines, exposure to tumor necrosis factor-α (TNF-α) also induced mucus expression both *in vitro* and *in vivo*[89-91].

Increased mRNA and protein expressions of hCLCA1 and IL-9, as well as mucus production, were detected in the bronchial biopsies samples from asthma, COPD and CF patients[44, 92-95]. Studies demonstrated that stimulation of mucosal tissue from upper airways of CF patients and primary normal human bronchial epithelial (NHBE) cells with Th2 cytokines significantly increased hCLCA1 and MUC5AC expression[39, 96]. In addition, intratracheal instillation of IL-13 in BALB/C mice induced 8- and 110-fold increases in MUC5AC and mCLCA3 mRNA expression, respectively. The same study also demonstrated that MUC5AC and mCLCA3 induction was abolished in Signal Transducer and Activator of Transcription (STAT)-6 knockout mice[97]. However, a lack of STAT6-binding sites in the MUC5AC promoter indicated that intermediate steps are required to convert the IL-13 signal to MUC5AC expression[98]. Soon after, the identity of consensus STAT6-binding sites in human and mouse CLCA gene promoter regions led to the hypothesis that activation of STAT6 induces CLCAs expression, and in turn induces mucin gene expression[57]. This hypothesis was confirmed by a recent study that IL-13-

induced hCLCA1 expression activated p38 (see section 2.3.3.2; MAPKs), which sequentially led to stimulation of MUC5AC expression (Figure 2.5)[41].

Interestingly, although studies have demonstrated that overexpression of mCLCA3 induced mucus expression both *in vitro* and *in vivo* model systems, a study reported that mCLCA3 knockout mice showed no difference in mucus production in the presence or absence of pro-inflammatory cytokines stimuli compared to control mice[99]. However, this could be attributed to the redundancy of CLCA genes in mice, in which other mCLCAs compensate for the loss of mCLCA3 function. Precedent exists for this redundancy, the expression of mCLCA5 was found to increase in mCLCA3 knockout mice exhibiting goblet cell metaplasia, and mCLCA5 gene transfer was shown to be sufficient to drive goblet cell metaplasia in mice[54].

Nonetheless, mCLCA3 or hCLCA1 remains to be a potential therapeutic target for goblet cell metaplasia. Recently, a study reported that mCLCA3 antibody could dampen the pathological symptoms in asthmatic mice[55]. Asthmatic mice treated with mCLCA3 antibody displayed a remarkable reduction in airway inflammation, the number of goblet cells and mCLCA3 expression in lung tissue[55].

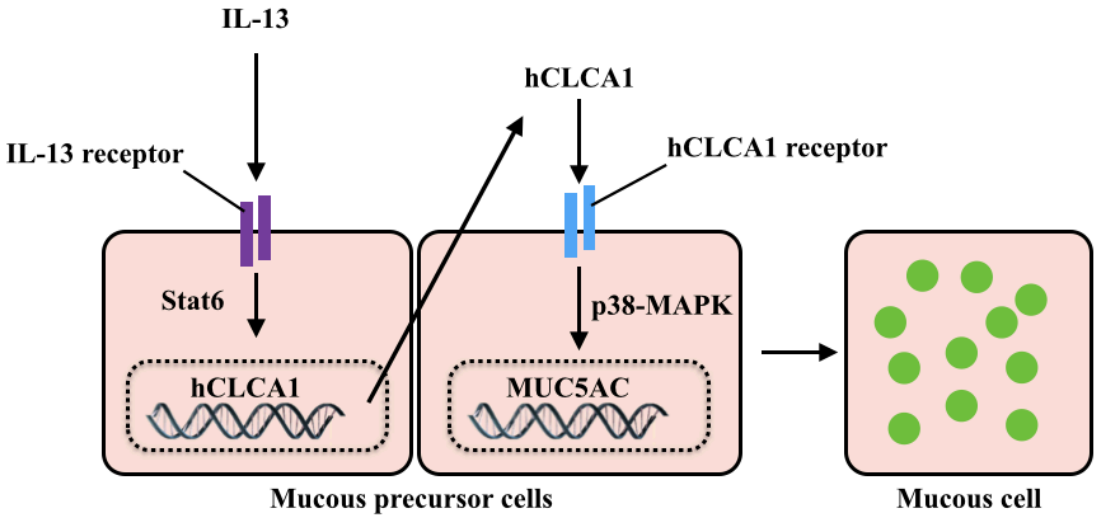


Figure 2.5. Schematic model of IL-13-induced hCLCA1 regulation of mucin gene expression. Interaction of IL-13 and IL-13 receptor leads to STAT6 activation and induction of hCLCA1 gene expression. The expressed hCLCA1 then secretes into the extracellular compartment and interacts with a putative receptor, and in turn activates p38 that subsequently leads to the production of MUC5AC gene expression. This eventually results in mucus production in airway mucous cell.

2.1.3.3 *Regulation of immune response*

Recently, we have identified the novel functional role of hCLCA1 as a signaling molecule to regulate immune response[45]. We showed that secreted hCLCA1 could induce pro-inflammatory expression in macrophages, and this effect applied to both cell line and primary cells. In our unpublished data, we demonstrated that the VWA domain of hCLCA1 was responsible for such induction in macrophage via the activation of NF-κB and MAPK pathways. Similar to the study that demonstrated hCLCA1 activated p38 to induce mucin gene expression[41], we also observed correlative activation of p38 in macrophages by the VWA domain of hCLCA1. Shortly after our findings that hCLCA1 could activate macrophages, another group reported that mCLCA3 was also able to modulate immune response during acute *Staphylococcus aureus* pneumonia[100]. This study illustrated that mCLCA3 knockout mice had a decrease in neutrophil infiltration into the bronchoalveolar space during bacterial infection, and a significant decrease in the mRNA and protein expression of IL-17 and IL-8. This study supports our findings that hCLCA1 could function as an immune regulator, which might explain the pleiotropic effect of hCLCA1.

2.2 Respiratory diseases

The most common chronic airway diseases are cystic fibrosis, asthma, and chronic obstructive pulmonary disease[101, 102]. The hallmarks of these diseases include goblet cell metaplasia, mucus hypersecretion, and airway inflammation[103-105]. High levels of hCLCA1 and pro-inflammatory cytokines are found in the airways of patients suffering from these diseases[44, 92, 95]. hCLCA1 has been implicated to play a role in mucus secretion, and pro-inflammatory cytokines (see section 2.3; Immune response) are responsible for initiating and sustaining inflammation in these airway diseases.

2.2.1 *Cystic fibrosis*

Cystic fibrosis is a life-shortening autosomal recessive disease that affects mainly the exocrine pancreas and airways, although it also affects other organs[106]. It is estimated to affect 1 in 2500-6000 births depending on the region and ethnic origin, with Caucasians having the highest rate[102, 107, 108]. Patients suffering from CF often present with abnormally viscous secretions in the airways of the lungs, leading to inflammation, tissue damage and organ destruction[103].

This disease is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is a cAMP-dependent anion channel expressed in the apical membrane of the airway epithelial cells[109, 110]. Normally, CFTR transports Cl^- from epithelial cells into the airway. This draws Na^+ to follow passively through a paracellular pathway to maintain electroneutrality. This causes water to move into the airways by osmosis, thus establishing airway surface liquid (ASL) homeostasis to hydrate the airways[111, 112]. Moreover, CFTR is also a known down-regulator of the epithelial sodium channel (ENaC), an amiloride-sensitive sodium channel that absorbs Na^+ into the cells, which functionally decrease the ASL[113]. This combined effect on Cl^- and Na^+ transport normally generates an osmotic force for water to move across the epithelium into the airways, establishing the ASL and allowing appropriate mucociliary clearance[112, 114]. Mucociliary clearance is a process in which mucus is removed from the respiratory epithelium by the beating of cilia.

However, the function of CFTR is altered due to mutations in the protein in CF. Absence or reduced function of epithelial CFTR leads to alteration of salt and water transport in the airways. Malfunction of CFTR causes decreased Cl^- secretion and increased Na^+ absorption, resulting in ASL volume depletion (Figure 2.6)[115, 116]. This depletion results in abnormal viscous secretions that can cause obstruction of airways in the lung, resulting in chronic respiratory infections, inflammation, bronchiectasis, and eventually respiratory failure[117, 118].

To date, over 1900 CFTR gene mutations associated with CF have been reported to the Cystic Fibrosis Mutation Database[119]. Mutations of CFTR can be grouped into 6 categories according to the mechanistic basis: absence of full length CFTR mRNA transcripts (Class I), abnormal folding and degradation in ER (Class II), disrupted regulation of channel gating (Class III), abnormality of chloride conductance (Class IV), decreased quantities of mature CFTR transcripts (Class V) and defect in stability of protein (Class VI)[120-124]. Out of all the mutations, the class II mutation with the deletion of a phenylalanine residue at the 508 position to be the most common in CF, accounting for ~70% of all CF chromosomes[123].

Although there is currently no cure for CF, there have been major advances in the management of CF that resulted in longer survival in the last few decades. The life expectancy of CF patients increases from 14 years in 1980's to 50 years at present in Canada[119]. Most commonly, CF patients use antibiotics to prophylactically suppress lung infection[125-127]. At present, researches in gene therapy and molecule therapy are being conducted in an attempt to cure CF. Gene therapy aimed at correcting the genetic alteration by inserting recombinant viral vectors to express functional CFTR, but the expression of the introduced gene was short-lasting[128]. On the other hand, there have been promising results in molecular therapy aimed at correcting the functional defect in the protein level. Clinical trials on a drug called ivacaftor has demonstrated improved lung function in CF patients carrying the G551D (Class III mutation) variant by increasing channel activity of both wild type and defective cell-surface CFTR potein[120, 129]. However, there is still a long way to go for CF treatment since there are over 1900 variants associated with CF, and the most common CF variants by far is not rescued by drugs such as ivacaftor.

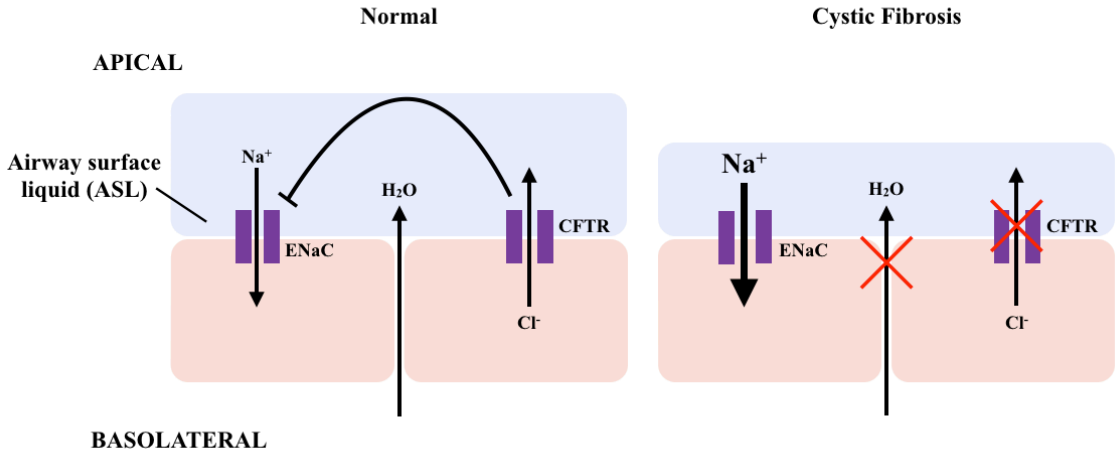


Figure 2.6. Overview of major ion channels involved in cystic fibrosis. This diagram represents the movement of Na^+, Cl^- and H_2O in normal and CF epithelial cells, as well as their respective transporters. The apical side corresponds to the airspace, where the ASL is secreted. Normally, CFTR transports Cl^- to the airways and down-regulates ENaC function, resulting in increased concentration of Na^+ in the ASL. This creates strong osmotic drive that draws water to the airways, facilitating airway hydration and mucociliary clearance. However, CFTR function is impaired in CF, resulting in increased absorption of Na^+ into the cells. This leads to airway dehydration (decreased in ASL) and mucus build up, contributing to the obstruction of airways in the lungs.

2.2.1.1 *Association of hCLCA1 with CF*

The first evidence showing an association of hCLCA1 with cystic fibrosis came from a study investigating the protein expression levels from upper airway tissues from CF patients[95]. The study reported that the expression of IL-9, IL-9R and hCLCA1 were increased in biopsy samples of nasal polyps, the nasal mucosa and sinus mucosa of CF patients. There was also an increase in periodic acid-Schiff stain (PAS)-staining, which indicates increased mucus producing cells in CF patients. Positive correlations were found between IL-9, IL-9R and hCLCA1 positive cells, as well as between mucus producing and hCLCA1 positive cells[95]. The same group subsequently demonstrated similar results from biopsies of bronchial mucosa of CF patients, further supporting the correlations between IL-9, hCLCA1 and mucus overproduction[94]. To explain the mechanism, the same group showed that stimulation with IL-4, IL-9 and IL-13 increased both mRNA and protein expression of hCLCA1 in mucosal tissue from upper airways of CF patients[96]. However, mucin protein and MUC5AC mRNA expression were not significantly changed in response to Th2 cytokine stimulation. One possible explanation for this observation is that mucosal tissue from CF patients already exhibited goblet cell metaplasia; therefore subsequent stimulation with Th2 cytokines had minimal effect on mucus expression in the tissues that were already expressing high levels of mucus. Lastly, one group performed an association study and reported that hCLCA1 was an important modulator for the DIDS-sensitive Cl^- secretion in the gastrointestinal tract of CF patients[46]. It suggested that hCLCA1 modulates an alternative chloride secretion pathway in CF patients. It is possible that hCLCA1 increases the Cl^- secretion through TMEM16A to compensate for the loss of CFTR function in the gastrointestinal tract, and the same mechanism might occur in the airways as well.

2.2.2 *Asthma*

Asthma is a chronic inflammatory airway disease characterized by reversible airflow obstruction, bronchial hyper-responsiveness, and airway inflammation[105]. The prevalence of asthma is estimated to be 5-10% in the general population, affecting more than 300 million people globally, with approximately 250,000 deaths annually[101, 130]. The symptoms of asthma includes cough, shortness of breath, wheezing, chest tightness and excess airway mucus production.

The exact cause of asthma is not known, but it is believed that genetic susceptibilities and environmental exposures are major factors. Multiple genetic polymorphisms have been associated with asthma development[131, 132], and parental history of asthma also increases asthma susceptibility[133]. Environmental factors that are associated with asthma pathogenesis include aeroallergen sensitization, respiratory viruses, early life microbial exposures, cigarette smoke, air pollution, diet and the host microbiome, vitamin D deficiency, antioxidants, and stress[134-142].

The hygiene hypothesis has been used to explain the increased rates of asthma since industrialization and urbanization (Figure 2.7)[143]. The hygiene hypothesis proposed that increased cleanliness and reduced family size reduced the exposure to bacteria and viruses in early childhood[144]. The biological basis behind this hypothesis is that Th1 cells immune response is not induced early in life due to improved sanitary environment[145]. Many bacteria and viruses elicit Th1-mediated immune response, which down-regulates Th2-mediated immune response (see section 2.3.1.2; Adaptive immune response)[146]. However, insufficient induction of Th1-mediated immune response renders the body to be more susceptible to induction of Th2-mediated immune response. This stimulates the antibody-mediated immunity of the immune system, which in turn leads to allergic disease such as asthma[136, 147].

Normally, acute inflammation is initiated within minutes of recognition of a danger signal, and it resolves itself within hours or days. However, in asthmatic patients, this naturally protective response becomes uncontrolled and leads to chronic changes to airway structure and function[148]. Airway inflammation inflicts damages to the airway epithelium, and this leads to airway remodeling (Figure 2.8). Airway remodeling is characterized by airway wall thickening, subepithelial fibrosis, and goblet cell metaplasia[149].
When the epithelium is injured, it releases profibrotic mediators to facilitate wound healing. However, the airway remodeling process persists in asthmatic patients because of the ongoing host immune responses that generate mediators[150]. The pathological consequence of this persistent 'repairing phase' is thickening of airway wall, which leads to airflow obstruction. The airflow obstruction is also intensified with the excess mucus production from the goblet cell metaplasia[151]. As mentioned above, asthma is triggered by stimulation of Th2-mediated immune response, and one of the Th2 cytokines secreted is IL-13. Studies have demonstrated that IL-13 is

required for goblet cell metaplasia, and this leads to elevated MUC5AC mucin gene expression[152, 153].

Beside airway wall thickening and goblet cell metaplasia, smooth muscle contraction is a prominent contributor to airflow obstruction. Excessive airway narrowing can lead to severe shortness of breath, respiratory failure and even death. Smooth muscle contraction can be physiologically induced by acetylcholine released from parasympathetic system or by histamine and leukotrienes released from mast cells or basophils. In healthy individuals, smooth muscle contraction only causes mild airway narrowing in response to these agonists. However, asthma patients suffer from bronchial hyper-responsiveness, which results in a substantial increase in sensitivity to these agonists. This is demonstrated by a dramatic increase in airway resistance[154]. Interestingly, a study has reported that there was an increase in the expression of calcium-activated chloride channel TMEM16A in airway epithelial cells of asthmatic patients. The same study demonstrated that increased TMEM16A expression contributed to mucus secretion and airway smooth muscle hyper-responsiveness[155]. As mentioned above, hCLCA1 increases CaCC current by stabilizing surface expression of TMEM16A. This reinforces the role of hCLCA1 in contributing to the pathologies of asthma.

Although there is currently no cure for asthma, symptoms can usually be controlled. Common medications include the use of bronchodilators, inhaled corticosteroids, oral leukotriene antagonist, or mast cell stabilizer. However, 5-10% of individuals with asthma respond poorly to asthmatic drugs, and these severe asthmatic patients are at a relatively high risk for fatal asthma attacks[156]. Numerous clinical trials have been conducted in the recent years, including continuous inhaled corticosteroid treatment, continuous or intermittent fluticasone treatment, prednisolone treatment, or a combination of long acting beta agonists (LABAs) with fluticasone treatment[157-161]. However, these treatments were short-lasting and the effects diminished once administrations of drugs were discontinued. In addition, LABAs also induce adverse effects in a small number of patients[162]. Continued research is required to generate new ideas and insights for asthma prevention and treatment.

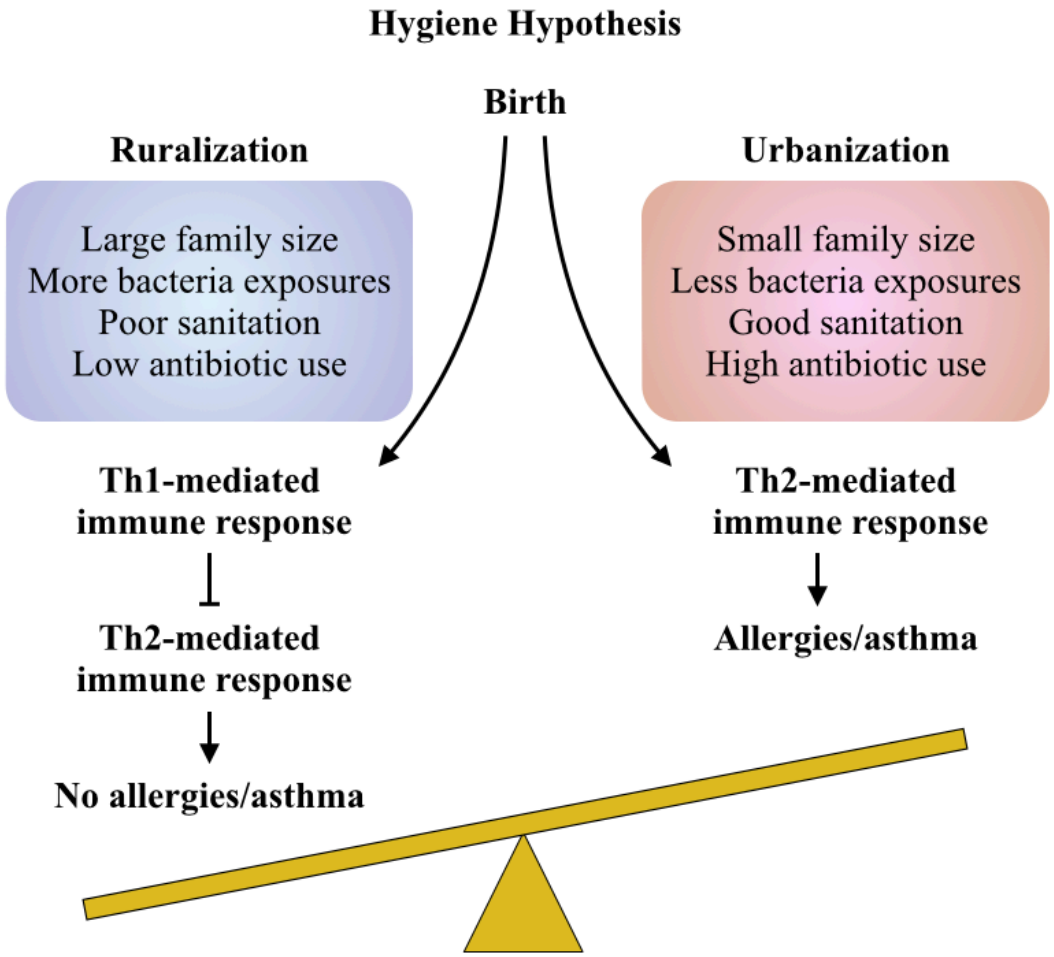


Figure 2.7. Overview of association between hygiene hypothesis and asthma. Increased rates of asthma have been reported since industrialization and urbanization in the past few decades. Although both genetic and environmental factors influence the pathogenesis of asthma, hygiene hypothesis is often used to explain the rise of prevalence of allergies or asthma. In urbanized area, insufficient stimulation in Th1-mediated immune response early in life predisposes the body to be more susceptible to stimulate Th2-mediated immune response, resulting in allergies or asthma.

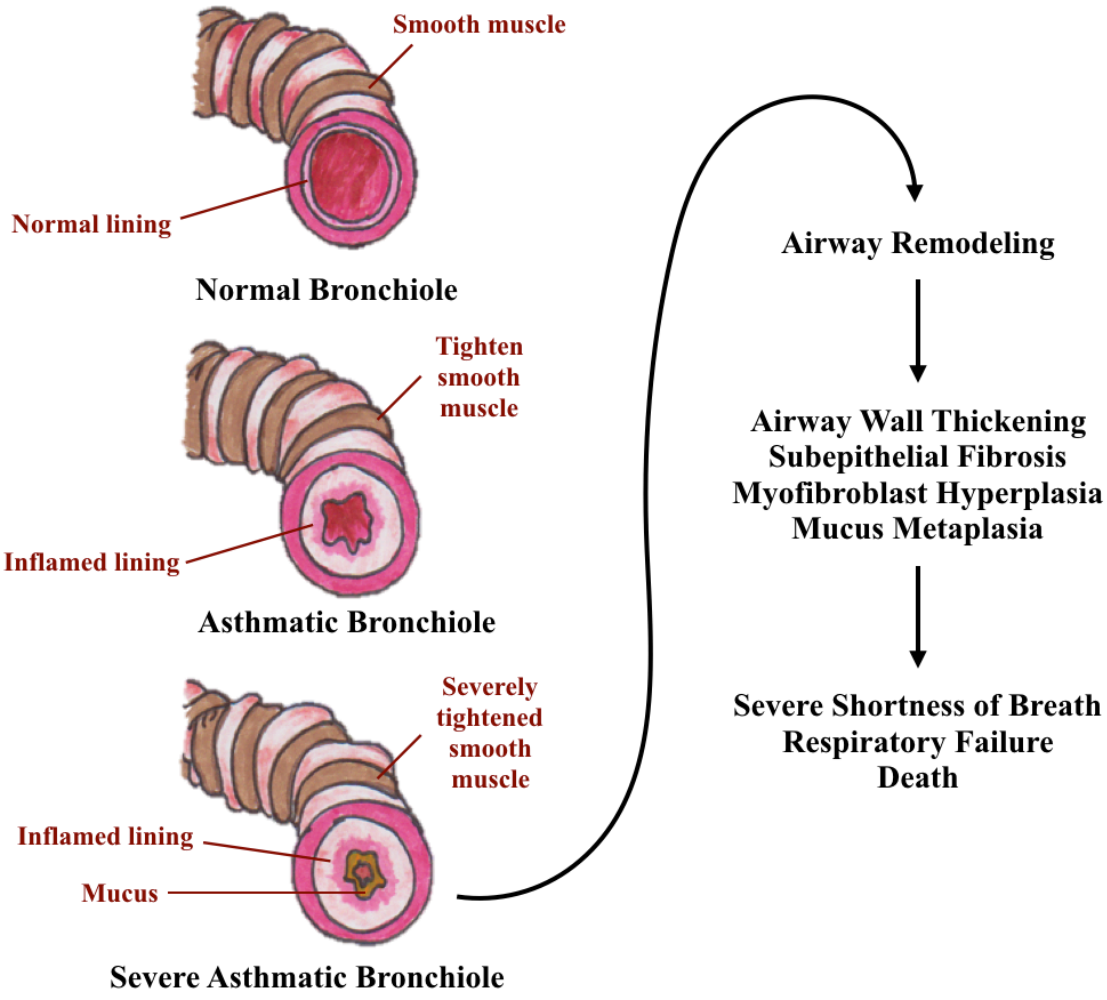


Figure 2.8. Schematic model of increasing severity in airway remodeling. In asthmatic patients, persisted airway inflammation leads to airway remodeling. The hallmarks of airway remodeling include airway wall thickening, subepithelial fibrosis, myofibroblast hyperplasia, goblet cell metaplasia, etc., resulting in severe airway obstruction. The pathophysiological consequences of severe airway obstruction can lead to severe shortness of breath, respiratory failure, and even death.

2.2.2.1 *Association of hCLCA1 with asthma*

The association of hCLCA1 or its murine ortholog mCLCA3 with asthma first came to light in 2001. The study identified mCLCA3 to be one of the most highly induced genes in the lungs of an asthma mouse model[43]. Intratracheal administration of adenovirus-expressing antisense mCLCA3 RNA effectively suppressed the asthma phenotype including goblet cell metaplasia and airway hyper-responsiveness. In addition, the asthma phenotype was worsened when mice were administrated an adenovirus-mediated overexpression of mCLCA3. *In vitro* experiments also showed that transient expression of hCLCA1 or mCLCA3 increased mucus production and MUC5AC expression in NCI-H292 mucoepidermoid cell line[43].

A hallmark of asthma is the overexpression of Th2 cytokines in the airways, which Th2 are sufficient to drive mucus over-production[86-88]. These cytokines also induce hCLCA1 or mCLCA3 expression both *in vitro* and *in vivo*[39, 85]. Those results demonstrated that hCLCA1 expression is mediated by Th2 cytokines, and suggested hCLCA1 as a potential regulator of mucus production. To further support this idea, bronchial biopsies samples from asthmatic patients exhibited increased the expression of IL-9, IL-9R and hCLCA1, and positive correlations were found between these genes and mucus producing cells[44]. Moreover, a recent study reported that administration of mCLCA3 antibody to asthmatic mice significantly reduced the development of asthma-associated clinical signs[55]. In addition, single nucleotide polymorphisms (SNP) in hCLCA1 have also been shown to affect patients' susceptibility to asthma[163].

2.2.3 *Chronic obstructive pulmonary disease*

Chronic obstructive pulmonary disease is defined as "a disease characterized by airflow limitation that is not fully reversible. The limitation is usually progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases" by the Global Initiative for Chronic Obstructive Lung Disease (GOLD). The World Health Organization (WHO) estimates 65 million people have moderate to severe COPD, and it is one of the leading causes of morbidity and mortality worldwide. The typical clinical manifestations of COPD are chronic bronchitis and emphysema[164]. Chronic bronchitis is a condition of chronic inflammation

and remodeling in bronchi that results in chronic cough and sputum production. Emphysema is a condition of lung parenchyma destruction and alveolar enlargement that results in loss of elastic recoil followed by decline in lung function and forced expiration volume. It is clinically accepted that chronic inflammation is linked to the lung tissue destruction and alveolar enlargement in COPD patients[165].

Currently, only one gene is identified to be a risk factor for COPD. Patients with alpha 1-antitrypsin (ATT) deficiencies are at higher risk for developing COPD[104, 166]. However, the primary risk factor for COPD development is cigarette smoking[167]. Upon exposure to cigarette smoke, respiratory epithelial cells undergo structural alterations that lead to squamous cell metaplasia, decreased mucociliary clearance, and loss of barrier function[168, 169]. Exposure to cigarette smoke also induces airway epithelial cells to release inflammatory mediators that result in chronic bronchitis[170]. Goblet cell metaplasia is one of the manifestations of chronic bronchitis, and excess mucus remains in the airway due to the reduced mucociliary clearance, leading to chronic cough, sputum production and infection.

As inflammation in the lung progresses, large numbers of macrophages and neutrophils infiltrate the lung parenchyma, leading to emphysema[171, 172]. The accumulation of these inflammatory cells serves as a self-perpetuating stimulus for further immune activation, leading to severe lung injury. Macrophages from COPD patients have been shown to secrete increased levels of IL-8, which further promotes neutrophil infiltration[173]. In addition, neutrophil phagocytosis by alveolar macrophages has been found to be impaired in COPD, resulting in persistent neutrophil infiltration in the lung[174].

A protease-antiprotease balance theory has been hypothesized to explain the development of emphysema[175]. This hypothesis proposes that under inflammatory environments such as exposure to cigarette smoke, neutrophils and macrophages secrete excess elastases and matrix metalloproteinases (MMP) which facilitate the destruction of lung parenchyma (Figure 2.9)[176, 177]. It is evident that elastase-deficient mice and MMP knockout mice displayed higher resistance to develop emphysema with chronic cigarette smoke exposure[178, 179]. Recent studies also supported the idea that emphysema is partly due to a decrease of endothelial cell maintenance

factors. One example of this is vascular endothelial growth factor (VEGF). It was reported that the expressions of VEGF and its receptor were significantly decreased in COPD patients, which promoted apoptosis of alveolar wall cells and emphesema[180, 181].

There is currently no cure for COPD, however, the clinical symptoms can be improved. Cessation of cigarette smoking is considered to be the most important aspect of COPD management. Although the decline of lung function persists after cigarette cessation, the rate of decline is reduced[182]. Other non-pharmacological approaches include reducing the risk for infection through vaccination against influenza or pneumococcus, pulmonary rehabilitation, and oxygen supplementation. Amongst pharmacological approaches, the primary medications are bronchodilators, which have been shown to significantly reduce COPD exacerbations[183]. Other pharmacological interventions include the use of antioxidants, anti-inflammatory drugs, and antibiotics. Although many non-pharmacological managements and pharmacological approaches are available to reduce COPD exacerbations, the degree of reduction is usually limited. Therefore, novel approaches are needed to prevent the progression of the disease.

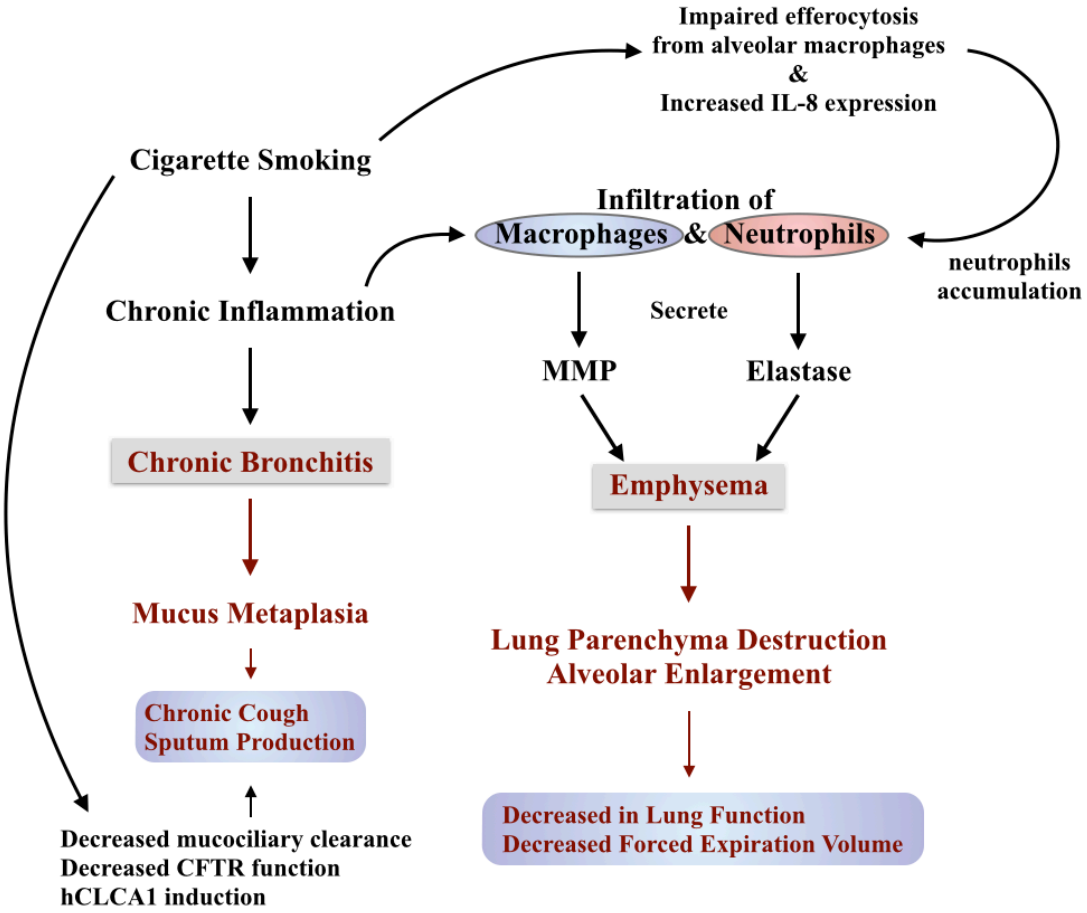


Figure 2.9. Impact of cigarette smoking in the development of COPD. Cigarette smoking is a primary factor for the development of COPD, and the clinical manifestations are chronic bronchitis and emphysema. Cigarette smoke exposure induces inflammation and facilitates macrophage and neutrophil infiltration. These inflammatory cells then secrete tissue-destroying enzymes to disintegrate alveolar wall, leading to emphysema. The pathophysiological consequences of emphysema include significant decrease in lung function and forced expiration volume.

2.2.3.1 *Association of hCLCA1 with COPD*

Although most of the studies on hCLCA1 have been focused on its association with asthma, several studies have linked hCLCA1 with COPD. Similar to asthma and CF, biopsies of bronchial samples from COPD patients displayed markedly increase in IL-9, IL-9R, hCLCA1 and mucus expression[92]. Increased expression of eCLCA1, equine ortholog of hCLCA1, was also observed in horses with recurrent airway obstruction. Recurrent airway obstruction is a common spontaneous disease in horses with high clinical, functional, and pathological similarities to human asthma and COPD[184, 185]. It is well-known that cigarette smoking is a primary risk factor for COPD development. A study demonstrated that expression of MUC5AC and rCLCA1, rat ortholog of hCLCA1, were up-regulated in rats exposed to cigarette smoke[84]. In the same study, the author also showed that expression of hCLCA1 and MUC5AC were up-regulated in H292 mucoepidermoid cell line exposed to smoke solution. Moreover, twenty-two SNPs were identified in hCLCA1 that were associated with COPD, and the author suggested that SNPs in hCLCA1 could be a useful indicator for COPD susceptibility[186].

2.3 Immune response

2.3.1 *Overview of innate and adaptive immune system*

The immune system is a collection of cells and proteins that functions to protect an organism from foreign antigens such as microbes, viruses, cancer cells, and toxins. The immune system can be classified into the innate immune system and the adaptive immune system. An adaptive immune response is called into action when the innate immune response has failed to eliminate the pathogen.

2.3.1.1 *Innate immune response*

The innate immune system constitutes the first line of defense against an intruding pathogen. It is a non-specific defense mechanism which is initiated within hours after the host encounters an antigen. The primary function of innate immunity is to trigger inflammation and recruit immune cells to sites of infection.

There are many conserved features of pathogens that are common to many pathogens but are absent in the host. These pathogen-associated molecular patterns (PAMPs) allow the host's immune system to distinguish between self and non-self antigens. For example, molecules found on the surface of many microorganisms do not occur in mammalian cells. These include the peptidoglycan cell wall and flagella of bacteria, as well as lipopolysaccharide (LPS) on Gram-negative bacteria and lipoteichoic acids on Gram-positive bacteria[187]. PAMPs are then recognized by the pattern recognition receptors (PRRs) expressed on the surface of immune cells such as resident macrophages, dendritic cells, neutrophils, and epithelial cells[188]. The most well-studied PPRs in humans are toll-like receptors (TLRs). Interaction of PAMPs with their specific TLRs induces the activation of NF-κB signaling and MAPK pathways, which causes secretion of pro-inflammatory cytokines[189]. Cytokine production also leads to the release of antibodies and other proteins and glycoproteins that activates the complement system. The complement system is part of the innate immune system that involves a series of biochemical cascades. There are three ways in which the complement system protects against infection. First, it generates activated

complement proteins to identify and opsonize foreign antigens, predisposing them to phagocytosis by phagocytes including macrophages or neutrophils. Second, the small fragments of some complement proteins act as chemoattractants to recruit more phagocytes to the site of infection. Third, complement proteins assemble a membrane-attack complex to create a pore in the lipid bilayer membrane of the bacteria to destroy membrane integrity[190].

After recognizing PAMPs, resident macrophages become activated and drive the influx of inflammatory leukocytes such as neutrophils and monocytes through chemokine secretion. Neutrophils are the earliest cells recruited to the site of inflammation. They phagocytose and then destroy pathogens in the phagosomes by producing reactive oxygen species and through the action of hydrolytic enzymes[191]. After ~48 hours, monocytes are recruited to the site of inflammation and differentiate into either macrophages or dendritic cells. The dendritic cells and macrophages are antigen-presenting cells (APCs) that phagocytose and process pathogens, and present processed antigens on their major histocompatibility complex (MHC) class II molecules to initiate an adaptive immune response[190]. In addition, macrophages are also responsible for eliminating aged neutrophils via a process called efferocytosis and initiate immune resolution (Figure 2.10)[192]. However, when foreign particles are still present, activated macrophages perpetuate inflammation by recruiting more leukocytes and secreting pro-inflammatory cytokines. During airway inflammation, pro-inflammatory cytokines and hCLCA1 were reported to be up-regulated in the airways[92, 193], and we demonstrated that hCLCA1 could act as a signaling molecule to induce pro-inflammatory cytokine expression in macrophages[45], thereby enhancing inflammation, and increasing immune cells infiltration. A recent study also reported that mCLCA3 knockout mice displayed decreased neutrophil infiltration in acute *Staphylococcus aureus* pneumonia[100]. Together, these studies further support the role of hCLCA1 in regulation of the immune system.

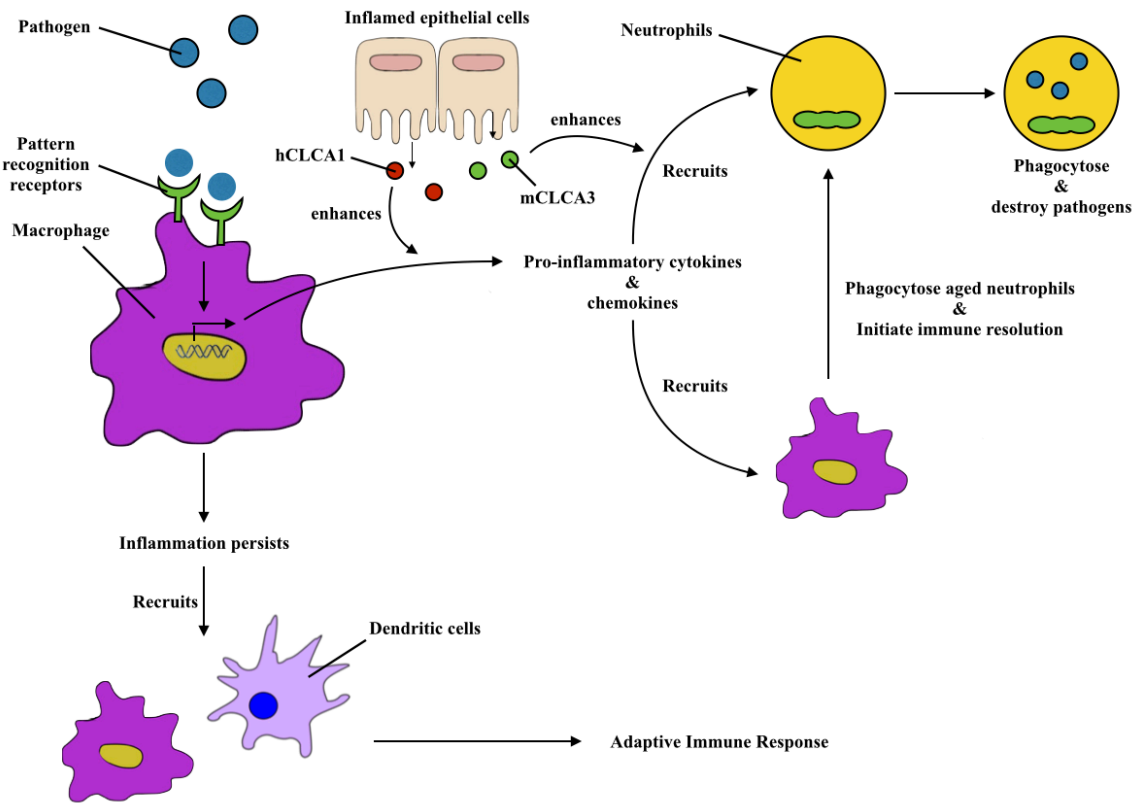


Figure 2.10. Overview of innate immune response. Macrophages become activated when they detected PAMPs through their PRRs. This induces macrophages to increase expression of pro-inflammatory cytokines and chemokines, leading to an influx of neutrophil infiltration to the site of inflammation. Neutrophils phagocytize and destroy pathogens through reactive oxygen species and hydrolytic enzyme. After 48 hours, additional macrophages are recruited to the site of infection to eliminate aged neutrophils and initiate immune resolution. If the inflammation persists, additional immune cells such as macrophages and dendritic cells will be recruited, resulting in the initiation of adaptive immune response. In addition, hCLCA1 or mCLCA3 secreted from inflamed epithelial cells also play a role in the innate immune response. Our work showed that hCLCA1 could induce pro-inflammatory cytokines expression in macrophages. Another study demonstrated that mCLCA3 was involved in neutrophil recruitment during bacterial infection in mice.

2.3.1.2 *Adaptive immune response*

Adaptive immunity is an antigen-dependent and antigen-specific mechanism that is called into action against pathogens that are able to evade or overcome innate immune defenses. The primary function of adaptive immunity is to eliminate specific pathogens or pathogen-infected cells and the development of an immunologic memory that can quickly eliminate the same pathogen in the future[194]. The main players of adaptive immunity include antigen-presenting cells (APCs), T cells, and B cells. These cells are all derived from hematopoietic stem cells in the bone marrow, and they are subsequently developed into different specific types based on different stimulants.

Pathogens are first ingested by antigen-presenting cells (APCs), which are usually dendritic cells, but also macrophages, B cells, fibroblasts and epithelial cells. APCs process the pathogens and subsequently present the antigens on major histocompatibility complex (MHC) molecules. MHC can be classified as either class I which are found on all nucleated cells, or class II which are only found on macrophages, dendritic cells and B cells. MHC class I molecules present endogenous antigens (cells infected with pathogens) while MHC class II molecules present exogenous antigens (phagocytosis of the pathogens). T cells become activated when their T-cell receptor (TCR) recognize an antigen-MHC complex on an APC. Binding of MHC class I molecules activate cytotoxic T cells while binding of MHC class II molecules activate T helper cells. Subsequently, activated T helper cells (Th2) are involved in differentiating B cells into either plasma cells or memory B cells.

2.3.1.2.1 *Cytotoxic T cells*

Activated cytotoxic T cells undergo clonal expansion in which they produce effector cells, and the effector cells search for pathogen-infected cells that have presented the antigens on the MHC class I molecules on the surface of the cells. Interaction of their TCRs and the MHC class I molecules on infected cells lead to the release of perforin, granzyme or granulysin to the infected cells, which results in either cell lysis or apoptosis. Upon resolution of the infection, most of the effector cells will die and be cleared by phagocytes. However, a subset of them are retained as

memory cells and can quickly differentiate into effector cells upon future encounters with the same antigen[194].

2.3.1.2.2 *T helper cells*

Naïve T helper (Th) cells are activated when their TCRs interact with MHC class II molecules. Although T helper cells have no cytotoxic or phagocytic activity, they mediate immune responses by directing other cells to eliminate pathogens. Once activated, naïve Th cells can differentiate into either Th1 or Th2 cells depending on the stimulant[195].

Activated Th1 cells are characterized by their capacities to produce interferon-gamma (IFN-γ), TNF-α and IL-12, which activate macrophages to kill microbes located within their phagosomes, activate cytotoxic T cells to kill infected cells, and induce B cells to make opsonizing and neutralizing antibodies. If naïve Th cells are differentiated into Th2 cells, they secrete IL-4, IL-5, IL-10 and IL-13 to defend the host against extracellular pathogens. Th2 cells can stimulate B cells to make most classes of antibodies, including immunoglobin (Ig)-E and some subclasses of IgG antibodies that bind to mast cells, basophils and eosinophils. Binding of IgE antibodies stimulates mast cells and basophils to release histamine, which increases the permeability of the capillaries to immune cells. Mast cells and eosinophils are responsible for initiating acute inflammatory responses such as allergy or asthma, and IgE antibodies are also associated with allergic reactions. These cells release local mediators that cause sneezing, coughing, or diarrhea and help expel extracellular pathogens from epithelial surfaces of the body. Similar to cytotoxic T cells, a few of Th cells remain as Th memory cells upon resolution of infection[194]. In general, Th1 responses are more effective against intracellular pathogens, whereas Th2 responses are more effective against extracellular pathogens such as parasites.

2.3.1.2.3 *B cells*

Unlike T cells, B cells can recognize pathogen via the antigen receptors on the surface of the B cells, and they ingest the pathogens using receptor-mediated endocytosis. The pathogens are then degraded, processed, and displayed on the MHC class II molecules of B cells. This attracts

activated Th2 cells to bind to the MHC class II molecules and provide cognate assistance, resulting in the B cell proliferation and differentiation into antibody-secreting plasma cells or memory B cells. Plasma cells produce pathogen-specific antibodies, which help eliminate the pathogens through the complement cascade. Memory B cells are long-lived B cells which can readily elicit an antibody-mediated immune response upon subsequent infections with the same pathogen (Figure 2.11)[194].

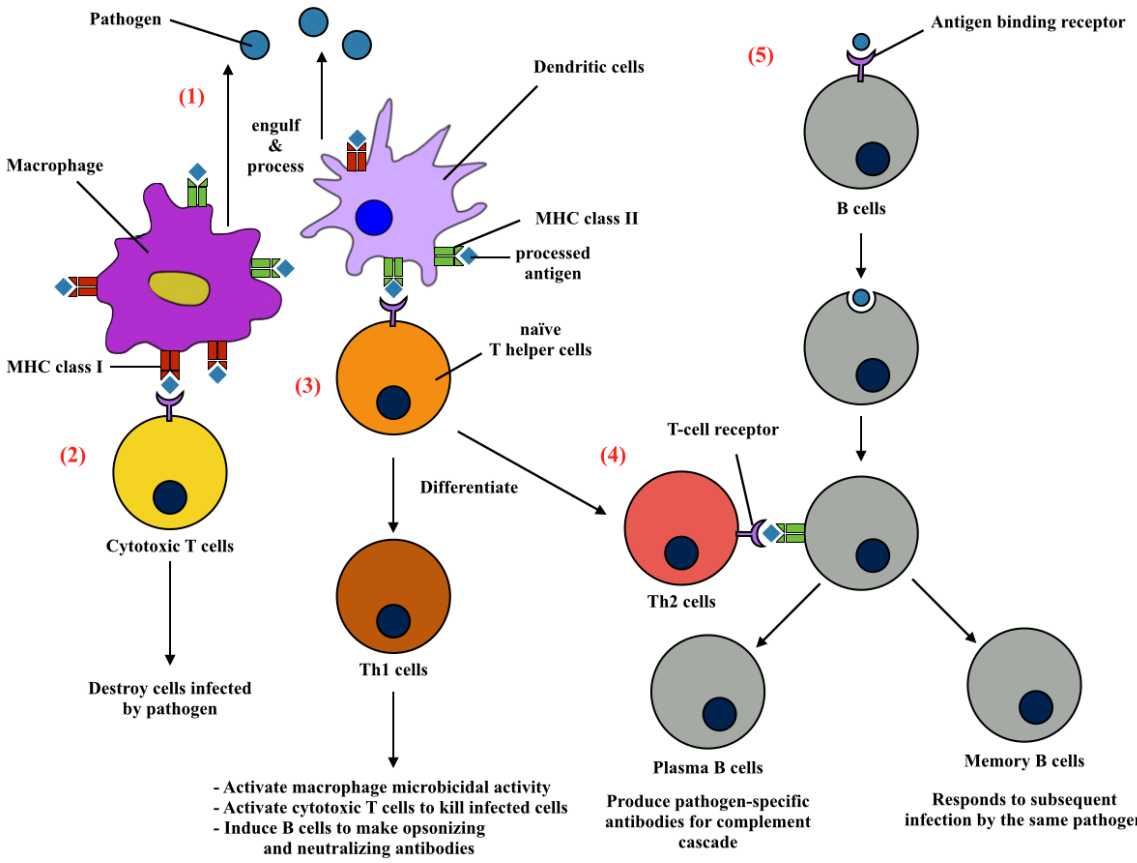


Figure 2.11. Overview of adaptive immune response. 1) Pathogens are first ingested by APCs such as macrophages and dendritic cells, then the pathogens are processed and presented on APCs with either MHC class I or MHC class II molecules. T cells become activated when their T-cell receptor interacts with the MHC complex on APCs. 2) Binding of MHC class I molecules activate cytotoxic T cells while 3) binding of MHC class II molecules activates naïve T helper cells. Activated cytotoxic T cells are responsible for destroying infected cells. Activated naïve T helper cells differentiate into either Th1 or Th2 cells. Th1 cells secrete cytokines to enhance the microbicidal activity of macrophages, enhance killing ability of cytotoxic T cells, and induce B cells to produce opsonizing and neutralizing antibodies. Whereas, 4) Th2 cells stimulate B cells to produce other subclasses of antibodies to attract different immune cells for the complement cascade. 5) When B cells detect pathogens through their antigen binding receptor, they ingest and process the pathogens and present them on their MHC class II molecules. This facilitates interaction with Th2 cells, which leads to proliferation and differentiation of B cells into either plasma cells or memory B cells. Plasma cells are responsible for producing antibodies to initiate the complement cascade to eliminate the pathogens, while memory B cells are responsible for preparing the host to elicit quick responses upon subsequent infection by the same pathogens.

2.3.2 *Cytokines in airway diseases*

Cytokines are important mediators that play roles in orchestrating the inflammation in airway diseases such as asthma and COPD by recruiting, activating, and promoting the survival of different inflammatory cells in the respiratory tract (Table 2.3). Generally, cytokines are categorized into four groups: Th1, Th2, pro-inflammatory, and anti-inflammatory cytokines. In these chronic airway diseases, Th1 and pro-inflammatory cytokines are responsible for initiating and sustaining airway inflammation. Th2 cytokines are suggested to contribute to mucus hypersecretion, airway hyper-responsiveness, and subepithelial fibrosis. On the other hand, anti-inflammatory cytokines are important for immune resolution.

Bronchial biopsies from asthmatic patients showed increased filtration of eosinophils, activated mast cells, and Th2 cells[196]. On the other hand, bronchial, small airways and lung parenchyma biopsies from COPD patients showed increased filtration of Th1 cells, neutrophils, and macrophages[197]. These inflammatory cells are largely responsible for the inflammatory phenotypes observed in asthma and COPD patients by secreting different cytokines. However, as the severity of these airway diseases worsens, this specific Th-mediated distinction (asthma is Th2 and COPD is Th1) becomes indistinguishable.

2.3.2.1 *Th1 cytokines*

IFN-γ is the predominant cytokine produced by Th1 cells that are highly expressed in the airways of COPD patients, but its expression is reduced in asthmatic patients[198, 199]. IFN-γ is an important mediator for inducing Th1 cytokines expression while suppressing the Th2 cytokines expression[200]. It also facilitates cytotoxic T cells and Th1 cell infiltration in the lungs by up-regulation of chemokines receptors and chemokines[201]. In addition, IFN-γ is also responsible for priming macrophages for microbicidal functions.

IL-12 is a key player in host defense by promoting the survival and growth of Th1 cells, and it is produced by activated macrophages, dendritic cells, and airway epithelial cells[202]. IL-12 has a crucial role in the induction of IFN-γ production by Th1 cells, as well as increasing cytotoxicity

of natural killer (NK) cells and cytotoxic T cells[203, 204]. Similar to IFN-γ, the expression of IL-12 is higher in COPD but not in asthmatic patients[198, 205].

IL-18 is a pro-inflammatory cytokines that is produced in macrophages. It was initially discovered as an IFN-γ-inducing factor, it was later found that IL-18 also stimulates the production of other pro-inflammatory cytokines, chemokines, and transcription factors[206]. IL-18 synergizes with IL-12 to activate T cells and NK cells for IFN-γ production, inhibition of IL-4-dependent IgE production, and the suppression of Th2 response. In individuals with COPD, IL-18 expression was increased in alveolar macrophages and cytotoxic T cells in the airways and is correlated with disease severity[207].

2.3.2.2 *Th2 cytokines*

IL-4 induces naïve Th cells to differentiate into Th2 cells. Upon activation by IL-4, Th2 cells subsequently produce additional IL-4 to facilitate Th2 cell proliferation in a positive feedback manner. It is an important mediator for isotype switching of B cells from producers of IgG to producers of IgE, which is associated with hypersensitivity and allergic reactions[193]. IL-4 also suppresses the differentiation of Th1 cells by antagonizing the production of IFN-γ and IL-12 in macrophages and Th1 cells. In addition, IL-4 is responsible for promoting wound-healing macrophages, resulting in fibrogenesis and tissue repair (see section 2.4.2.2.1; wound-healing macrophage)[208]. IL-4 has also been shown to induce mucus production, and together with fibrogenesis of wound-healing macrophages, contributing to the airway remodeling of asthma patients[86].

Similar to IL-4, IL-9 is highly induced in individuals suffering from airway diseases such as CF, asthma, and severe COPD[44, 92-95]. A study reported that overexpression of IL-9 in mice induced eosinophil infiltration, mucus hyperplasia, mastocytosis, airway hyper-responsiveness, and increased expression of other Th2 cytokines and IgE[209]. Another study showed that many of these effects were mediated by IL-13, and overexpression of IL-9 in IL-13 knockout mice did not induce mucus production and pulmonary eosinophil infiltration[210].

IL-13 has been attracting a lot of attentions as a therapeutic target for asthma treatment because it has been shown to induce airway hyper-responsiveness, mucus metastasis, airway smooth muscle proliferation, and subepithelial fibrosis[211]. Similar to IL-4, IL-13 also promotes macrophages to differentiate into wound-healing macrophages. In addition, like other Th2 cytokines, IL-13 expression was increased in airways of asthmatic patients, whereas it was reduced in patients with severe COPD[212]. As mentioned above (see section 2.1.3.2; hCLCA1's association with mucus expression), studies reported that IL-13 induced the expression of hCLCA1 through STAT6 pathway, and hCLCA1 subsequently induced MUC5AC gene expression through activation of p38[41], demonstrate the role of hCLCA1 in the pathology of asthma.

2.3.2.3 *Pro-inflammatory cytokines*

Pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β were increased in the sputum and bronchoalveolar lavage (BAL) fluid in asthmatic and COPD patients[193]. Although monocytes and macrophages are the main sources of these cytokines, they are also produced by activated lymphocytes, endothelial cells, and fibroblasts[213]. Expression of these pro-inflammatory cytokines is in part mediated through activation of the NF-κB and MAPK pathways.

TNF-α is one of the first pro-inflammatory cytokines to be released in response to pathogens. It induces vasodilation and increased vascular permeability, facilitating infiltration of lymphocytes, neutrophils, and monocytes. It also helps recruitment of these immune cells to the site of inflammation by regulating chemokine release[213]. TNF-α is suggested to play a critical role in amplifying inflammation via activation of the NF-κB pathway in asthmatic patients in a positive feedback manner[214]. In addition, TNF-α promotes classical activation of macrophages (see section 2.4.2.1; classical activation of macrophage), which perpetuate inflammation by releasing increased levels of pro-inflammatory cytokines including IL-1β, IL-6, and additional TNF-α[215].

IL-1β is mainly produced in monocytes and macrophages. It is a potent pro-inflammatory cytokine that induces the release of chemokines and vascular adhesion molecules, promoting monocyte infiltration in early inflammation[216, 217]. IL-1β also induces expression of itself in newly recruited monocytes, thus reinforcing the overall process[218]. IL-1β expression was reported

to increase in the airways of asthmatic patients, and it initiated the production of other pro-inflammatory cytokines. In individuals with COPD, IL-1β was reported to activate macrophages to secrete inflammatory cytokines, chemokines, and MMP[173]. As mentioned in previous section, MMP is responsible for destruction of lung parenchyma by degrading extracellular matrix (ECM)[176].

IL-6 is a pleiotropic cytokine that has both pro-inflammatory and anti-inflammatory functions. It provides a link between innate and adaptive immunity by promoting differentiation of B cells into plasma cells and activating cytotoxic T cells[213]. Similar to TNF-α and IL-1β, IL-6 is responsible for recruiting monocytes to the site of inflammation[219]. However, IL-6 also plays a role in anti-inflammation by inhibiting the production of TNF-α and IL-1β[220]. Consistent with other pro-inflammatory cytokines, increased expression of IL-6 was found in the sputum of both asthmatic and COPD patients[221, 222]. IL-6 might play a role in controlling the degree of inflammation in chronic airway diseases.

IL-8, also known as neutrophil chemotactic factor, is a potent chemoattractant for neutrophils that is produced mainly in activated macrophages. IL-8 levels were markedly increased in the sputum of COPD and asthma patients, and its expression is correlated with the numbers of neutrophils in the sputum[223, 224].

2.3.2.4 *Anti-inflammatory cytokines*

IL-10 is a potent anti-inflammatory cytokine that is mostly produced by monocytes and regulatory macrophages. IL-10 inhibits the production of many pro-inflammatory cytokines that are overexpressed in asthma and COPD, by interfering with the activation of NF-κB and MAPK pathways[225-227]. IL-10 suppresses the expression of MHC class II molecules in activated macrophages, indirectly suppressing the activation of T helper cells[226]. In addition, macrophages exposed to IL-10 exhibited reduced microbicidal activity and their capacities to respond to IFN-γ[228, 229]. However, expressions of IL-10 were decreased in individuals with asthma and COPD[230, 231].

Table 2.3. Key cytokines involved in asthma and COPD.

Cytokines	Expression in asthma	Expression in COPD	Main functions
Th1 cytokines			
IFN-γ	Decreases	Increases	Induces expression of Th1 cells Suppresses expression of Th2 cells Primes macrophages for microbicidal activity
IL-12	Decreases	Increases	Induces IFN-γ production in Th1 cells
IL-18	Decreases	Increases	Induces IFN-γ production in Th1 cells Inhibits IL-4-dependent IgE production
Th2 cytokines			
IL-4	Increases	Decreases	Facilitates Th2 cells proliferation Promotes isotype switching of B cells Antagonizes IFN-γ and IL-12 production
IL-9	Increases	Decreases	Induces eosinophil infiltration Induces mucus hypersecretion Increases expression of other Th2 cytokines
IL-13	Increases	Decreases	Induces airway hyper-responsiveness Increases hCLCA1 expression Promotes wound-healing macrophages
Pro-inflammatory cytokines			
TNF-α	Increases	Increases	Facilitates immune cells infiltrations Promotes classical activated macrophages Amplifies inflammation
IL-1β	Increases	Increases	Promotes monocyte infiltration Induces macrophages to induce inflammatory response
IL-6	Increases	Increases	Promotes differentiation of plasma cells Activates cytotoxic T cells Facilitates monocyte infiltration
IL-8	Increases	Increases	Recruits neutrophils to the site of inflammation
Anti-inflammatory cytokines			
IL-10	Decreases	Decreases	Inhibits the production of pro-inflammatory cytokines Interferes NF-κB and MAPK pathways Suppresses the expression of MHC class II molecules in macrophages Suppresses the activity of T helper cells

2.3.3 *Signal transduction pathways in airway diseases*

2.3.3.1 *Nuclear factor-kappa B*

Nuclear factor-kappa B is viewed as a master regulator of the inflammatory response because it plays a pivotal role in regulating the immune response (Figure 2.12). It is involved in a major regulatory signal transduction pathway that regulates genes responsible for both the innate and adaptive immune response. NF-κB usually exists as a heterodimeric complex of p50 and p65 subunits[232]. NF-κB is normally sequestered in the cytoplasm with an inhibitory protein called inhibitory κB (IκB)-α, which masks the nuclear translocation signal and prevents NF-κB from translocating into the nucleus. Following exposure to NF-κB inducers such as TNF-α, IL-1β, LPS and oxidative stress, IκB-α become phosphorylated and subsequently results in ubiquitination and degradation[233]. This allows the translocation of free NF-κB into the nucleus, where it binds to the promoter of target genes and regulates their transcription. Activation of NF-κB leads to up-regulation of many genes including chemokines, immune receptors, vascular adhesion molecules, stress response genes, regulators of apoptosis, transcription factors, growth factors, and pro-inflammatory cytokines such as IL-8, IL-1β, TNF-α, IL-6[234-237].

Activated NF-κB has been reported in individuals suffering from asthma, COPD, and CF, and it was implicated to contribute to the pathogenesis of these airway diseases[238-240]. Studies demonstrated that p50 knockdown mice display reduced pulmonary inflammation and airway hyper-responsiveness after ovalbumin challenge, and this was because p50 knockdown mice were deficient in producing chemokines that are responsible for recruiting eosinophils and T cells to the sites of inflammation[241, 242]. It was later confirmed by other groups that activation of NF-κB within airway epithelium was essential to induce airway inflammation in asthmatic mice[243, 244]. In the case of COPD, oxidative stress generated by cigarette smoking was reported to induce inflammation through activation of NF-κB and by inducing the release of pro-inflammatory mediators such as IL-8 and IL-1β[245]. Furthermore, a study reported that IL-8 was significantly induced in the BAL fluid of CF patients, and this IL-8 induction was attributed to the activation of NF-κB[246]. These results agree with our novel identified function of hCLCA1 as an inflammatory mediator. hCLCA1 is one of the most induced genes in airway diseases, and our

works have demonstrated that it could activate NF-κB pathway to induce pro-inflammatory cytokines. This is further supported by another study that concluded mCLCA3, murine ortholog of hCLCA1, mediated neutrophil infiltration via up-regulation of IL-8[100], and it is likely to be NF-κB-dependent as well.

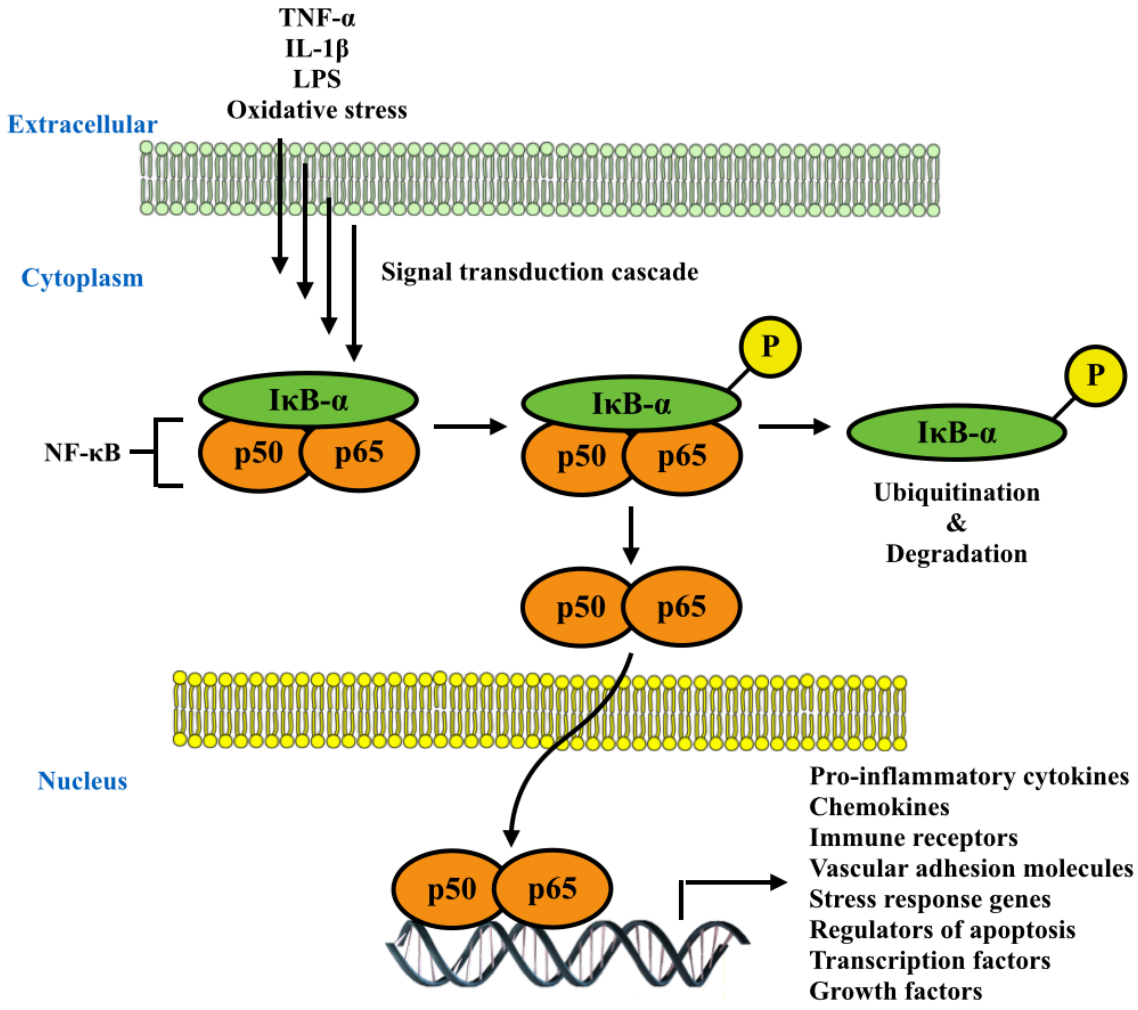


Figure 2.12. Brief overview of NF-κB pathway. NF-κB (heterodimer of p50 and p65 molecules) is normally sequestered in the cytoplasm by IκB-α. Upon exposure to stimuli such as TNF-α, IL-1β, LPS, and oxidative stress, IκB-α becomes phosphorylated and subsequently becomes ubiquitinated and degraded. This allows free NF-κB to translocate into the nucleus and bind to the promoters of target genes. Activation of NF-κB induces transcription of pro-inflammatory cytokines, chemokines, immune receptors, vascular adhesion molecules, stress response genes, regulators of apoptosis, transcription factors, and growth factors.

2.3.3.2 *Mitogen-activated protein kinases*

Mitogen-activated protein kinases (MAPKs) are important components of signaling modules that are involved in acute inflammatory responses in airway diseases. MAPKs are classified into three groups: the extracellular signal regulated kinase (ERKs), p38, and the c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPKs) (Figure 2.13). Upon exposure to stimuli, MAPKs are converted into activated forms via phosphorylation cascade, allowing their interactions with cytoplasmic substrates and translocations to the nucleus, where many MAPK targets, such as transcription factors and histones, are located[247]. The ERK pathway is stimulated by G-protein coupled receptors and growth factors, resulting in gene expression involved in proliferation, differentiation, and survival. The p38 and JNK pathways are activated mainly by cytokines, leading to gene expression involved in inflammation and apoptosis[248]. Studies have reported that patients with airway diseases such as CF, asthma, and COPD demonstrated increased phosphorylated forms of MAPKs, therefore, MAPKs are suggested to play an important role in mediating airway and lung inflammation[249-251].

Activation of ERK typically induces the expression of genes involved in proliferation, differentiation, and growth. However, studies have demonstrated that ERK also played a role in mediating inflammation by regulating the expression of different pro-inflammatory cytokines such as IL-6, IL-8, and IL-1β[252]. A recent study demonstrated that an ERK inhibitor was sufficient to suppress the expression of pro-inflammatory cytokines in H292 mucoepidermoid cells exposed to cigarette smoke condensate[253]. Beside ERK, p38 is also activated in COPD patients. Activation of p38 was reported to stabilize the mRNA of many pro-inflammatory cytokines and chemokines involved in COPD pathogenesis, and inhibition of p38 was reported to sufficiently attenuate the disease severity in animal models of COPD[254, 255]. This agrees with a subsequent study demonstrating inhibition of p38 pathway reduced the expression of TNF-α, IL-6, and IL-1β[256]. Similar to COPD, inhibition of ERK and p38 reduced the stability of IL-8 mRNA in cystic fibrosis lung epithelial cell line, while inhibition of JNK had no effect[251]. In the case of asthma, there was a positive correlation between disease severity and the amount of phosphorylated ERK and phosphorylated p38, as well as between phosphorylated ERK and the number of infiltrated eosinophils and neutrophils in the airways[249]. It is important to note that

activation of ERK and p38 have been shown to subsequently activate NF-κB pathway. In our work, we have demonstrated that hCLCA1 induced expression of pro-inflammatory cytokines via activation of ERK, p38, and NF-κB pathway. It is possible that hCLCA1 initially activates ERK and p38, which subsequently activates NF-κB to regulate inflammation.

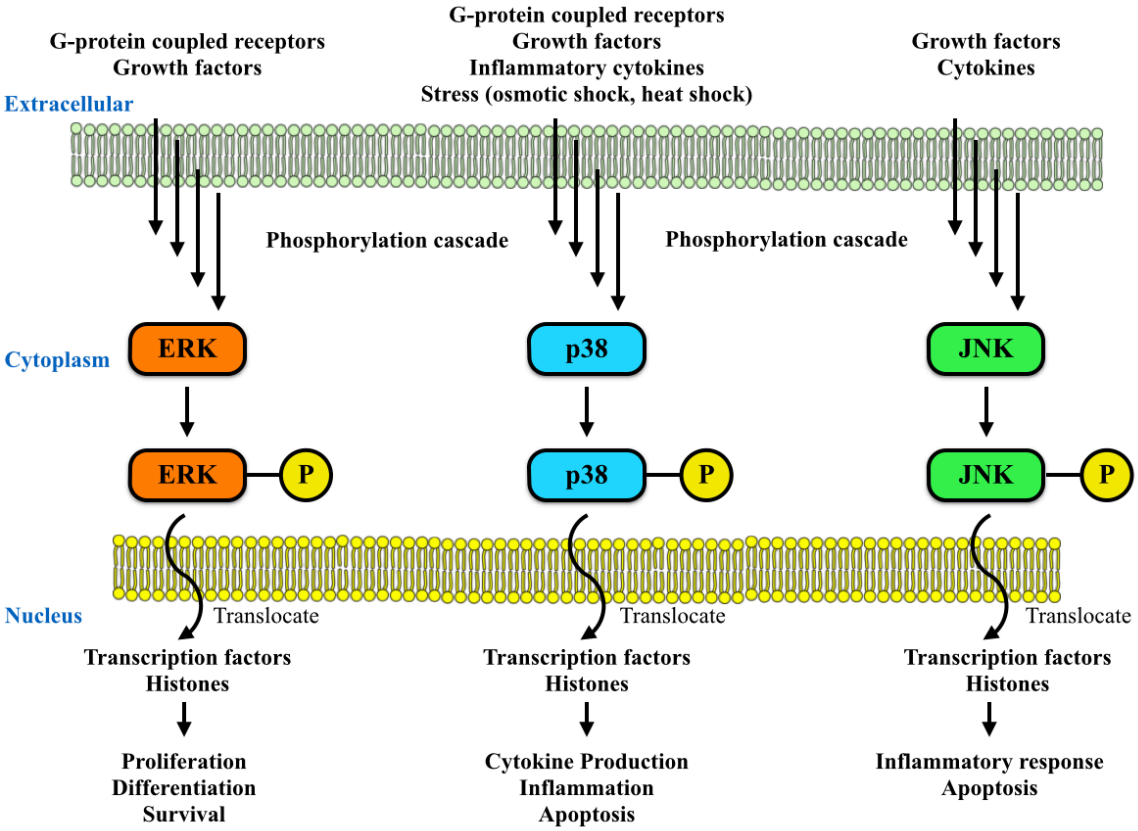


Figure 2.13. Brief overview of MAPK pathways. Upon exposure to stimuli, MAPKs (ERK, p38, and JNK) are activated via phosphorylation cascade. Activated MAPKs translocate into the nucleus and activate MAPK targets including transcription factors and histones. Activation of ERK pathway leads to gene expression involved proliferation, differentiation, and survival. Activation of p38 pathway induces gene expression involved in cytokine production, inflammation, and apoptosis. Last but not the least, activation of JNK pathway leads to gene expression responsible for inflammatory response and apoptosis.

2.4 Macrophage – biology and function

2.4.1 *Origin and Recruitment*

Macrophages are a type of immune cells that are located throughout the body. They phagocytose foreign particles, dead cells and debris and recruit additional neutrophils and macrophages in response to inflammatory signals. They are also one of the most important antigen-presenting cells (APCs) regulating the immune response in the body. They are differentiated from circulating monocytes, which migrate into tissue in both inflammatory and non-inflammatory states[257]. Monocytes are derived from hematopoietic stem cells in the bone marrow, which are also precursors of neutrophils, eosinophils, basophils, macrophages, dendritic cells and mast cells. Haematopoietic stem cells sequentially develop into monocytes, in which they are released from the bone marrow into the bloodstream.

During inflammation, monocytes are recruited to the site of inflammation via chemotaxis. Chemokines can be generated from damaged cells, pathogens, or resident macrophages at the site of inflammation. Circulating monocytes then extravasate through the endothelium of a blood vessel and enter the site of inflammation. Monocytes quickly differentiate into macrophages, and it was shown that the majority of the recruited macrophages displayed inflammatory characteristics[258].

2.4.2 *Mode of activation*

Macrophages can be activated into two different groups depending on stimuli: classically activated macrophages and alternatively activated macrophages. Alternatively activated macrophages can further be classified into two sub-populations: wound-healing macrophages and regulatory macrophages (Figure 2.14). Macrophages are highly plastic; i.e., how they are activated determines their functions. This characteristic allows macrophages to reversibly switch between different phenotypes depending on the microenvironment.

2.4.2.1 *Classical activation*

Classically activated macrophages play a role as effector cells in Th1-mediated immune response. These macrophages have enhanced microbicidal capacity and are able to secrete high levels of pro-inflammatory cytokines and mediators[259]. A combination of two signals is required to classically activate macrophages, and they are IFN-γ and TNF. IFN-γ is secreted from other immune cells during stress or infection, and this cytokine primes macrophages to secrete pro-inflammatory cytokines. This promotes macrophages to produce increased amounts of superoxide anions and oxygen and nitrogen radicals to increase their microbicidal ability[260]. However, TNF or inducer of TNF is required as a second signal in order for full activation. Although exogenous TNF can act as the second signal, but toll-like receptor ligand such as LPS is a more physiologically relevant agonist. Macrophages induce the expression of TNF when exposed to LPS, and the expressed TNF can cooperate with IFN-γ to activate macrophages in an autocrine manner[215].

When macrophages are classically activated, they secrete high levels of pro-inflammatory cytokines such as TNF, IL-12, IL-1 and IL-6. They also secrete increased levels of chemokines such as interferon gamma-induced protein 10 (IP-10), macrophage inflammatory protein 1α (MIP-1α), and monocyte chemotactic protein 1 (MCP-1) to recruit additional macrophages to the site of inflammation to sustain the inflammation[215]. Although classical activated macrophages are vital components of host defense, the cytokines and mediators they produced can also lead to host-tissue damage.

2.4.2.2 *Alternative activation*

2.4.2.2.1 *Wound-healing macrophages*

As the name suggests, wound-healing macrophages are responsible for wound-healing, angiogenesis, and ECM deposition. The major signals that promote wound-healing macrophages are IL-4 and IL-13[208]. Basophils and mast cells are important early sources of IL-4 following

tissue injury[261]. The wound-healing macrophage population is sustained by a Th2-mediated immune response, in which the signature cytokines are IL-4 and IL-13.

Contrary to classically activated macrophages, wound-healing macrophages fail to present antigen, produce little pro-inflammatory cytokines, and are inefficient at killing intracellular pathogens[262]. However, these cells secrete components of the extracellular matrix and contribute to tissue repair. Wound-healing macrophages produce high levels of fibronectin and transforming growth factor-β1 (TGFβ1), which promote fibrogenesis from fibroblastoid cells[263]. They also display enhanced arginase activity that leads to polyamine and proline biosynthesis, promoting cell growth, collagen formation and tissue repair[264]. Similar to classically activated macrophages, dysregulation of their matrix-repairing ability in wound-healing macrophages can lead to tissue fibrosis. Accumulating evidence suggests that these macrophages may contribute to the airway remodeling of asthmatic patients[265].

2.4.2.2.2 *Regulatory macrophages*

Regulatory macrophages arise in the later stages of adaptive immune responses, and the primary role for these cells is to dampen the immune response and limit inflammation[266]. Two signals are required for macrophages to induce their anti-inflammatory activity. The first signal can be one or more of the following: immune complexes, prostaglandins, adenosine or apoptotic cells, and has little or no stimulatory function on its own. However, when combined with a second signal, such as TNF-α or TLR agonist, the two signals induce the transformation of macrophages into regulatory macrophages.

The main role of regulatory macrophages is immune resolution. Unlike wound-healing macrophages, regulatory macrophages do not contribute to the production of the extracellular matrix[262]. Instead, these cells down-regulate IL-12 production while inducing the production of IL-10. IL-10 is an anti-inflammatory cytokine that inhibits the production and activity of various pro-inflammatory cytokines[267]. A study demonstrated that regulatory macrophages from IL-10 null mice fail to rescue LPS-challenged mice from lethal endotoxemia due to impaired immune resolution[268]. Like other types of macrophage populations, dysregulation of regulatory

macrophages can impose a detrimental effect on the host. Excess IL-10 can predispose the host to infection while insufficient IL-10 can result in tissue damage due to unresolved inflammation as seen in the mice with endotoxemia.

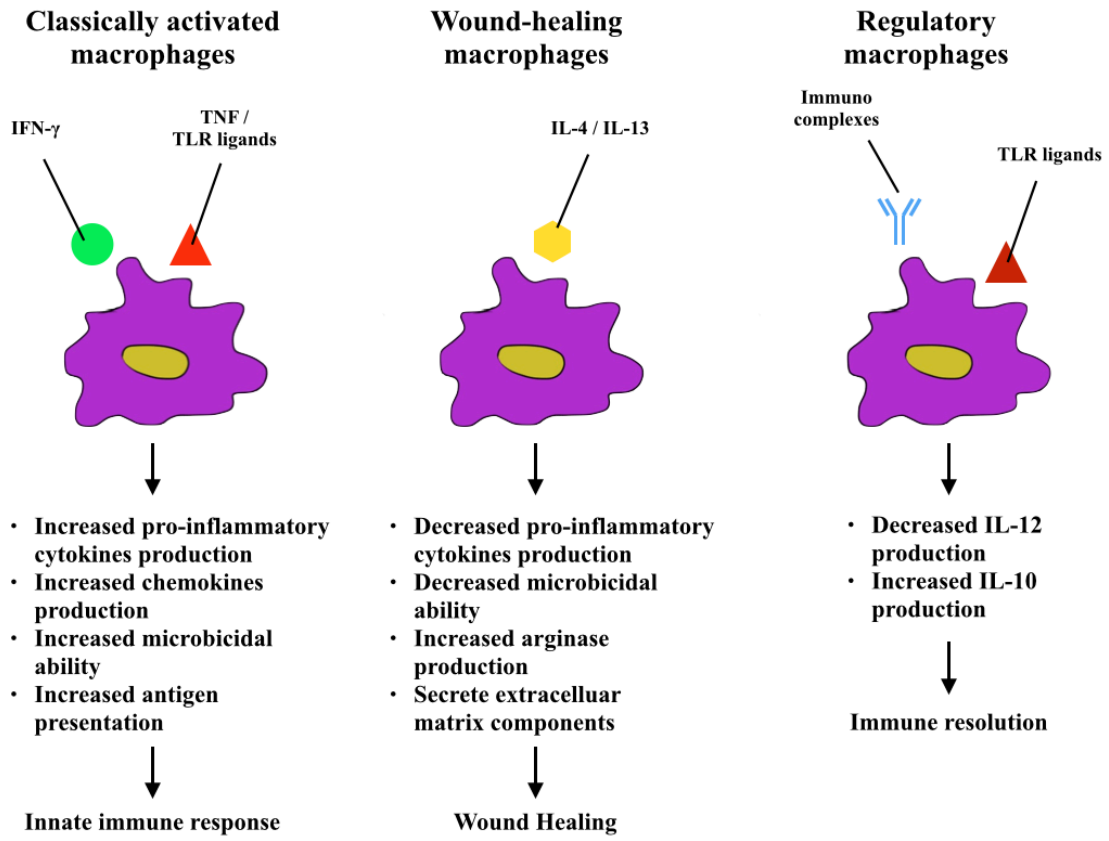


Figure 2.14. Mode of macrophage activation. Macrophages can be differentiated into different phenotypes depending on the stimuli. When classically activated with IFN-γ and TNF/TLR ligands, they are responsible for the innate immune response. When they are exposed to IL-4 or IL-13, they differentiate into wound-healing macrophages and are responsible for tissue repair. Lastly, macrophages differentiate into regulatory macrophages when stimulated with immuno-complexes (or prostaglandins, adenosine or apoptotic cells) and TLR ligands, and they are responsible for immune resolution.

2.4.3 *Alveolar macrophages*

2.4.3.1 *Origin and Maintenance*

Alveolar macrophages are monocyte-derived macrophages that localize between the epithelial surfaces and fluid linings of the alveoli and airways. They are highly specialized macrophages that function primarily in lung defense against inhaled particulate matter, microbes and environmental toxins. In the first few days after birth, fetal monocytes are colonized in the airways, forming alveolar macrophages via stimulation with granulocyte macrophage colony-stimulating factor (GM-CSF) secreted from epithelial cells in the lungs[269]. Alveolar macrophages have a high capacity for self-renewal, and local proliferation is the main means for alveolar macrophage to replenish themselves throughout life[270]. It was evident that alveolar macrophages were capable of replenishing themselves through proliferation when there was a partial depletion of alveolar macrophages during influenza infection[271]. Only in the case of substantial depletion of alveolar macrophages caused by radiation do haematopoietic stem cells-derived circulating monocytes contribute to alveolar macrophages repopulation[270].

2.4.3.2 *Role of alveolar macrophages in the lungs*

During homeostasis, alveolar macrophages remain in a relatively quiescent state with a regulatory-like phenotype, with roles primarily in tissue homeostasis and resolution of inflammation[272]. They produce low levels of inflammatory cytokines and have a reduced phagocytic ability compared to the resident macrophages in other tissues[273]. Also contrary to their counterparts, alveolar macrophages are inefficient at inducing T cell antigen-specific response because they have poor antigen presenting ability as well as a lack of expression of co-stimulatory molecules[274]. This is an important characteristic of alveolar macrophages so that pro-inflammatory responses are not triggered when exposed to tissue debris or innocuous antigens. They also produce immunosuppressive prostaglandins and anti-inflammatory transforming growth factor-β1 (TGFβ1) to suppress T cell activation[275]. An *in vivo* study has demonstrated that mice depleted of alveolar macrophages exhibited excessive inflammation and immunity to innocuous antigens[276].

The regulatory phenotype of alveolar macrophages is maintained by the lung microenvironments. Alveolar macrophages activation is tightly controlled through cell-cell and soluble mediator interactions, and the most important regulations are via interactions with OX-2 membrane glycoprotein (CD200) and IL-10. Alveolar macrophages express high levels of CD200R, and they interact with the CD200 expressed on type II alveolar epithelial cells, inhibiting the alveolar macrophages from pro-inflammatory activation[277, 278]. The regulatory phenotype of alveolar macrophages is also contributed by interaction with IL-10. The main sources of IL-10 are from healthy airway epithelium and alveolar macrophages during homeostasis[279]. As mentioned earlier, IL-10 is an anti-inflammatory cytokines that can suppress expression of pro-inflammatory cytokines[267]. This suggests that the destruction of airway epithelium or the loss of exposure to the regulatory ligands may direct alveolar macrophages to respond to airway antigens in a pro-inflammatory manner. As a result, this promotes macrophages to switch into different subtypes (classical, wound-healing, or regulatory) depending on the stimulants.

Although alveolar macrophages are essential to prevent unwanted inflammation from exposure to innocuous antigens, they are able to initiate strong immune responses to something more pathogenic. Alveolar macrophages express a wide array of receptors that recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). Activation of toll-like receptor (TLR) by PAMP inhibits the IL-10R signal transduction and overrides the inhibitory effect of IL-10[280]. Once activated, alveolar macrophages display greater phagocytic capacity, remove T cell suppression and produce increased levels of pro-inflammatory cytokines[281-283]. Activated alveolar macrophages also drive the influx of inflammatory leukocytes such as neutrophils and monocytes through chemokines secretion. The importance of alveolar macrophages in initiating inflammatory response is illustrated by a study that reported reduced chemokine production and neutrophil infiltration during inflammation due to alveolar macrophages depletion in mice[284].

2.4.3.3 *Alveolar macrophages in airway diseases*

Alveolar macrophages are one of the most important immune cells in the lungs that play a vital role in host defense and immune regulation during airway inflammation or infection. However,

they also contribute to some of the pathologies during airway diseases (Figure 2.15). Alveolar macrophages from CF patients displayed pro-inflammatory phenotypes and impaired phagocytosis ability[285], and they secrete high levels of chemokines in the absence of pulmonary infection[286]. Their inefficient clearance of pathogens and persistent release of pro-inflammatory cytokines perpetuate inflammation in the airways and eventually lead to tissue damage in CF patients.

Similar to CF, alveolar macrophages also contribute to the pathological lesions in COPD patients. As mentioned above, cigarette smoke exposure induced IL-8 expression and impaired efferocytosis in alveolar macrophages[174, 176], resulting in persistent neutrophil infiltration in the airways. Together with neutrophils, alveolar macrophages secreted excess elastases and MMPs that contributed to structural degradation (emphysema) of the lungs[176, 177]. This is salient when depletion of alveolar macrophages prevented the development of emphysema in an experimental model of COPD[287].

In the case of asthma, alveolar macrophages are suggested to contribute to airway remodeling. They are differentiated into wound-healing macrophages due to the stimulation of Th2 cytokines in the environment. They over-express profibrotic factors and display increased biogenesis of polyamines and proline to facilitate fibrogenesis of the airways[288, 289]. Alveolar macrophages from asthmatic patients secrete higher levels of IL-13, which perpetuate their wound-healing phenotype in an autocrine manner[290]. However, alveolar macrophages remain vital to asthmatic patients as their depletion worsens airway hyper-responsiveness and inflammation in asthmatic models *in vivo*[291, 292].

Alveolar macrophages are like double-edge swords. They are crucial to maintain pulmonary homeostasis by performing anti-inflammatory functions and clearing harmful pathogens, but they are also an integral part of the mechanisms perpetuating inflammation and tissue injury associated with different airway diseases.

The human hCLCA1 and its murine ortholog mCLCA3 are highly expressed from airway epithelial cells in airway diseases in inflammatory airway diseases in which alveolar macrophages are central to disease progression, such as cystic fibrosis, asthma, and COPD[43, 84, 95].

As mentioned above, alveolar macrophages regulate the immune system in the lung by controlling the expression of cytokines. During airway inflammation, pro-inflammatory cytokines are secreted from alveolar macrophages and other immune cells to eliminate pathogens. However, excess pro-inflammatory cytokines often lead to prolonged inflammation that can result in tissue damage. Our studies demonstrated that hCLCA1 is able to positively simulate the expression of different pro-inflammatory cytokines in alveolar macrophages. This suggests that hCLCA1 is partly responsible for the cytokine response elicited by alveolar macrophages in airway diseases.

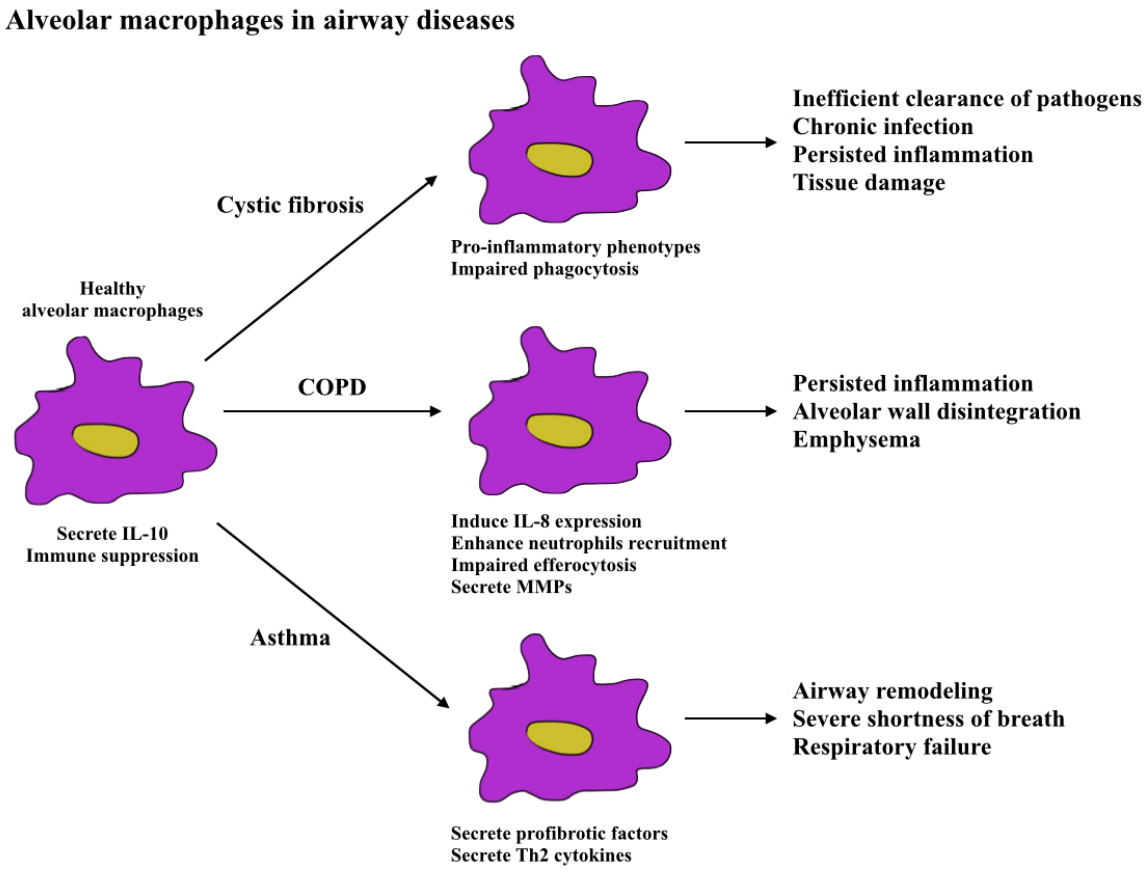


Figure 2.15. Schematic models of how alveolar macrophages play a role in airway diseases.

STUDY 1 – SECRETED HCLCA1 IS A SIGNALING MOLECULE THAT ACTIVATES AIRWAY MACROPHAGES

The *CLCA* gene family produces both secreted and membrane-associated proteins that modulate ion-channel function, drive mucus production and have a poorly understood pleiotropic effect on airway inflammation. The primary up-regulated human CLCA ortholog in airway inflammation is hCLCA1. Here we show that this protein can activate airway macrophages, inducing them to express cytokines and to undertake a pivotal role in airway inflammation. In a U-937 airway macrophage–monocyte cell line, conditioned media from HEK 293 cells heterologously expressing hCLCA1 (with or without fetal bovine serum) increased the levels of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α and IL-8). This effect was independent of the metalloprotease domain of hCLCA1. Primary porcine alveolar macrophages were similarly activated, demonstrating the effect was not cell line dependent. Similarly, immuno-purified hCLCA1 at physiologically relevant concentration of ~100 pg/mL was able to activate macrophages and induce pro-inflammatory response. This cytokine response increased with higher concentration of immuno-purified hCLCA1. These findings demonstrate the ability of hCLCA1 to function as a signaling molecule and activate macrophages, central regulators of airway inflammation.

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3.1 Introduction

CLCA genes (*CL* stands for chloride-channel modulating and *CA* for calcium-activated) are induced in airway epithelial cells by inflammation[14]. This induced expression often exceeds that of most other inflammatory mediators[293]. The gene products have a pleiotropic effect, generating secreted and membrane-associated proteins that increase mucus production, leukocyte infiltration and airway hyper-responsiveness, with single-nucleotide polymorphisms increasing asthma susceptibility as well[14, 43, 92, 163, 293-295]. Although CLCA proteins were originally identified as calcium activated chloride channels, we and others concluded that they only modulated channel pores (suggesting a signaling ability)[1, 4, 36, 59, 60]. How putative signaling ligands could cause a seemingly pleotropic effect on airway inflammation was unclear. One possibility was that CLCAs modified a central mediator of airway inflammation, such as the airway macrophage.

Airway macrophages are one of the major resident immune cell types responsible for lung defense[296]. How a macrophage is activated will determine its function[215]. "Classically" activated macrophages secrete high levels of pro-inflammatory cytokines (such as IL-8, IL-6 and IL-1β), enhancing their microbicidal capacity by producing oxygen and nitrogen free radicals[215]. "Alternatively" activated macrophages secrete high levels of anti-inflammatory cytokines (such as IL-10 and IL-12), dampening the immune response and promoting wound healing[297]. Generally, macrophage activation has a receptor-driven signal transduction mechanism requiring activation of (cytoplasmic membrane) potassium and chloride channels to proceed[298-300]. The primary up-regulated human CLCA ortholog in human airway inflammation is hCLCA1. If this protein activates macrophages, the pleiotropic effect of *CLCA* genes can be explained.

In this report, we used a human monocyte cell line (U-937) and primary porcine alveolar macrophages to test whether the secreted form of hCLCA1 can activate macrophages. We also assessed the role of the autoproteolytic metalloprotease (hydrolase) domain of hCLCA1, which contains a zinc-reactive HEXXH motif that cleaves the protein into a large ~90 kDa N-terminal and a small ~40 kDa C-terminal fragment, in the macrophage

activation process[68, 301]. We found using progressively purified secreted hCLCA1 protein, that it possesses an intrinsic ability to signal and activate airway macrophages. This signaling property was independent of its hydrolase domain activity.

3.2 Materials and Methods

3.2.1 *Cell culture and transfection*

Human embryonic kidney cells (HEK293 cell line; CRL1573; ATCC) were grown in DMEM-Glutamax medium (10566-016; Life Technologies) supplemented with 10% fetal bovine serum (FBS; 16000-044; Life Technologies) and 1% penicillin-streptomycin (pen-strep; 15140-122; Life Technologies) at 37 °C in a humidified atmosphere with 5% CO_2. Cells were seeded and transfected in six-well plates with the vectors pIRES2-EGFP, wild-type pIRES2-EGFP-hCLCA1 or hydrolase-inactive E157Q mutant pIRES2-EGFP-hCLCA1 (GenScript) using the transfection reagent FuGENE HD (E2311; Promega) at a 3:1 FuGENE HD Transfection Reagent to DNA ratio.

Human monocytes (U-937 cell line; CRL1593.2; ATCC) were grown in RPMI-1640 medium (SH3025502; Thermo Scientific) supplemented with 10% heat inactivated FBS and 1% pen-strep at 37 °C in a humidified atmosphere with 5% CO_2. Porcine alveolar macrophages were grown in RPMI-1640 medium supplemented with 20% heat inactivated FBS and 1% pen-strep at 37 °C in a humidified atmosphere with 5% CO_2.

3.2.2 *Media collection, immunoprecipitation and protein concentration determination*

At Day 2 post-transfection, conditioned FBS-containing medium was collected. Conditioned FBS-free medium was collected at Day 3 (after replacing the initial HEK293 medium with FBS-free DMEM at Day 2). Macromolecules in the collected media were concentrated using Amicon Ultra-15 Centrifugal Filter Units (UFC903008; EMD Millipore). The concentrations of the proteins were determined using a Bradford protein assay (500-0201; Bio-rad) Conditioned FBS-free hCLCA1 and FBS-free eGFP macromolecule samples were immunoprecipitated with a Pierce Crosslink Magnetic IP/Co-IP Kit (88805; Thermo Scientific) using hCLCA1-N14 antibody (sc-46866; Santa Cruz) according to manufacturers'

protocols. To improve the yield of the immunoprecipitation, we increased: the antibody amount used in the antibody coupling step to 8 µg from 5 µg, the antibody coupling time to 30 minutes from 15 minutes, and the sample incubation time to 1.5 h from 1 h. The concentration of the immunoprecipitated hCLCA1 protein was determined from a standard curve generated using a 2-fold dilution series of lysozyme (L-6876; Sigma-Aldrich) on a silver stained SDS-PAGE gel. The concentrations of lysozyme used in the standard curve were 100 pg/µL, 50 pg/µL, 25 pg/µL, 12.5 pg/µL, 6.25 pg/µL, and 3.125 pg/µL.

3.2.3 *Monocyte differentiation and activation*

Monocyte cells were seeded in each well (1.3×10^6 to 1.5×10^6 cells/well) in a 6-well plate and differentiated into macrophages with 0.1 nM phorbol-12-myristate-13-acetate (PMA; P8139; Sigma-Aldrich) in supplemented FBS-free RPMI-1640 medium for 18 h. The cells were washed 2 times with FBS-free RPMI-1640 medium and incubated in supplemented RPMI-1640 media containing 3, 6 or 10% FBS. In the conditioned FBS-containing medium experiment, 0.1, 1 or 10 mg/mL of eGFP or hCLCA1 was added to macrophages in 10% FBS growth medium. In conditioned FBS-free medium experiment, 66.7 µg/mL or 200.0 µg/mL of eGFP or hCLCA1 was added to macrophages in 3, 6, or 10% FBS growth medium to determine optimal FBS concentration; and 3.3 µg/mL, 16.7 µg/mL or 33.3 µg/mL of hCLCA1 was added to macrophages in 6% FBS growth medium to determine the dose response. In a further experiment, 45 µL of immuno-purified hCLCA1 (93.3 pg/mL or 141.7 pg/mL) or 45 µL of control (immunoprecipitation of eGFP using hCLCA1-N14 antibody) were added and incubated in 6% FBS growth medium for 24 h or 48 h.

3.2.4 *SDS-PAGE and western blot Analysis*

Cell lysates were collected using M-PER mammalian protein extraction reagent (78503; Thermo Scientific) with the addition of Halt protease and phosphatase inhibitor cocktail (78440; Thermo Scientific). The samples (cell lysates or media) were boiled in 2x denaturing buffer (20% glycerol, 4% SDS, 125mM Tris pH 6.8, 0.3mM bromophenol blue) and 10% β-mercaptoethanol (BME; M6250; Sigma-Aldrich), and they were analyzed by

10% or 12% SDS-PAGE. The SDS-PAGE gel was stained with coomassie blue stain (staining – 45% methanol, 10% glacial acetic acid, 45% water, 3g/L Coomassie Brilliant Blue R250; destaining – 20% methanol, 10% glacial acetic acid, 70% water) and a Pierce Color Silver Stain Kit (24597; Thermo Scientific) according to manufacturers' protocols. Densitometry was performed using a ChemiDoc MP System (170-8280; Bio-rad).

For western blot analysis, proteins were electroblotted onto PVDF membrane (RPN303LFP; GE Healthcare Life Sciences) with transfer buffer (25mM Tris, 192mM glycine, 20% methanol). Membranes were blocked overnight at 4 °C with 5% bovine serum albumin in PBST buffer (137mM NaCl, 2.6mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4, 0.1% Tween-20; Sigma-Aldrich) and subsequently probed for 2 h at room temperature with primary antibodies in PBST. The membranes were then incubated for 1 h at room temperature with secondary antibodies in PBST. Proteins were detected and analyzed using Typhoon Trio and ImageQuant TL system (63005583; GE healthcare Life Sciences). Densitometry analysis of intracellular IL-1β protein was normalized to GAPDH in each sample as previously described[302, 303]. The primary antibodies used were hCLCA1 (N-14; sc-46866; Santa Cruz), GAPDH (FL-335; sc-25778; Santa Cruz), IL-1β (H-153; sc-7884; Santa Cruz), and GAPDH (MAB374; EMD Millipore). The secondary antibodies used were Alexa Fluor 488 Donkey anti-Goat IgG antibody (A11055; Life Technologies), ECL Plex Goat anti-Mouse IgG-Cy5 antibody (PA45009; Amersham Biosciences), and DyLight 488 conjugate Goat anti-Rabbit IgG antibody (35552; Thermo Scientific).

3.2.5 *Bio-Plex suspension array system*

After 48 h activation with immuno-purified proteins, macrophage medium was collected and analyzed for cytokine levels using Bio-Plex Suspension Array System (171-000201; Bio-rad) according to the manufacturers' protocols. The Bio-Plex Pro Human Cytokine 8-plex Assay (M50-000007A; Bio-rad) we used included the following cytokines: IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFN-γ, and TNF-α.

3.2.6 *RNA isolation and real-time quantitative PCR*

For macrophages activated in FBS-containing media, RNA was extracted after 1, 2, 4, 6, 12 and 24 h using TRIzol Reagent (15596018; Life Technologies) according to manufacturers' protocols. For macrophages activated in FBS-free media, RNA was extracted after 24 h. For macrophages activated with immunoprecipitated proteins, RNA was extracted after 24 or 48 h. The collected RNA was analyzed with a GoTaq 2-Step RT-qPCR system (A6010; Promega) and Mx3005P real-time PCR machine (401514; Agilent). cDNA of each sample was measured in duplicates in Mx3005P real-time qPCR machine, and the average C_T (cycle threshold) value was used to calculate the fold difference of each gene. Human primers were designed for GAPDH, TNF-α, IL-12a, IL-8, IL-1β, IL-6 and IL-10; and porcine primers were designed for NADH dehydrogenase, TNF-α, IL-12a, IL-8, IL-1β, IL-6 and IL-10 (Table 3.1). hGAPDH and pNADH were used as the reference genes for analysis.

3.2.7 *Porcine alveolar macrophage isolation*

A solution of 100 mL of 0.1 M PBS+ (supplemented with 1% pen-strep and 1x antibiotic-antimycotic (15240112; Life Technologies)) was injected into porcine lungs. The lungs were obtained with specific permission for use in this study from a local federally inspected abattoir (Friesen's Meat Processing, Warman, SK). Lung lavage fluid was collected and filtered through 70 μm cell strainers (352350; Corning). The filtered cells were subjected to repeated centrifugation at 400*g* for 10 min at 4 °C and washed with 20 mL 1.0 M PBS+, 2–3 mL of erythrocyte lysing solution (155mM NH_4Cl, 10mM $NaHCO_3$, 1mM EDTA, pH 7.4 with HCl) for 20 s, 45 mL of 1.0 M PBS+ and then 25 mL of 1.0 M PBS+. The pelleted cells were subsequently re-suspended in culture medium (RPMI-1640 with 20% heat-inactivated FBS, 1% pen-strep and 1x antibiotic-antimycotic). The cells were counted using trypan blue (15250061; Life Technologies), and 1.5×10^7 cells/mL were cryopreserved in liquid nitrogen using culture medium with 10% DMSO.

3.2.8 *Porcine alveolar macrophage stimulation*

A total of 1.5×10^7 cells were thawed at 37 °C, added to 8 mL warm culture medium and centrifuged at 500*g* for 5 min. The cells were then re-suspended, and 1.5×10^7 cells were seeded in each well of a six-well plate with 3 mL of culture medium in each well. The plates were incubated 15–18 h at 37 °C under a humidified 5% CO_2 atmosphere. The cells were washed 2 times with culture medium and incubated in 3 mL culture medium. To activate the porcine alveolar macrophages, 66.7 µg/mL, 200 µg/mL or 1000 µg/mL of eGFP or hCLCA1 in FBS-free medium was added.

3.2.9 *Efficiency and fold difference calculations*

Dilution series from 1×10^0-fold to 1×10^{-5}-fold of cDNA were used to determine the primer efficiency. The C_T value obtained in each dilution was used to generate a linear plot of C_T vs. log copies. The efficiency of the primer set was determined with the equation $Eff = 10^{(-1/\text{slope})}$. The fold difference between hCLCA1- and eGFP-activated samples was determined using an efficiency-corrected calculation with eGFP-activated macrophage serving as control and hGAPDH or pNADH serving as the reference gene[304]:

$$ratio = \left(Eff_{\text{target}}\right)^{\Delta C_{\text{T, target}}\left(\text{Mean control} - \text{Mean sample}\right)} / \left(Eff_{\text{ref}}\right)^{\Delta C_{\text{T, ref}}\left(\text{Mean control} - \text{Mean sample}\right)}$$

3.2.10 *Statistics*

All data are expressed as means ± standard error of the mean (SEM). Each biological replicate was a result of an individual transfection paired with an eGFP transfection. Each biological replicate was performed on different days. Fold differences were calculated by comparing hCLCA1-activated macrophage to its paired eGFP-activated macrophage control. The normality test was done using Shaprio-Wilk test, and the fold difference values of RT-qPCR and Bio-Plex assay were analyzed using Kruskal-Wallis one-way analysis of variance test with Conover-Inman test. Friedman two-way analysis of variance was performed to compare the fold difference of mRNA expression between groups (time, wild type hCLCA1

vs. mutant hCLCA1, concentration, etc.). All western blot data was normalized to the appropriate controls, and each western blot was a result of an individual biological replicate. One-way t-tests were performed with the mean set as 1[305, 306]. Significance was determined at $p < 0.05$.

3.2.11 *Ethics Statement*

Obtaining and use of animal tissue was specifically approved for this study by the University of Saskatchewan's Institutional Animal Care and Use Committee (IACUC) Permit # 20120100.

Table 3.1. Primers used in RT-qPCR experiments.

Human qPCR Primers		
Gene Name	**Forward Primers (5′ → 3′)**	**Reverse Primers (5′ → 3′)**
GAPDH	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG
TNF-α	TGCTGCACTTTGGAGTGATCG	TGCTACAACATGGGCTACAGG
IL-12a	CAGTGGAGGCCTGTTTACCATTG	TACTACTAAGGCACAGGGCCATC
IL-8	TCTCTTGGCAGCCTTCCTGATTTC	ATTTCTGTGTTGGCGCAGTGTG
IL-1β	GCTGATGGCCCTAAACAGATG	TGTAGTGGTGGTCGGAGATTC
IL-6	AGCCACTCACCTCTTCAGAAC	GTGCCTCTTTGCTGCTTTCAC
IL-10	AAGCTGAGAACCAAGACCCAGACA	AAAGGCATTCTTCACCTGCTCCAC
Porcine qPCR Primers		
Gene Name	**Forward Primers**	**Reverse Primers**
NADH	TCATCGGGGCCCTACGAGCA	GGCGAAAGGTCCGGCTGCAT
TNF-α	ACGCTCTTCTGCCTACTGCACTTC	TCCCTCGGCTTTGACATTGGCTAC
IL-12a	CCACTTGAACTAGCCACGAATGAG	AGATACTGCTAAGGCACAGGGTTG
IL-8	AGGACCAGAGCCAGGAAGAGAC	CTTGCCAGAACTGCAGCCTCAC
IL-1β	CTCCAGCCAGTCTTCATTGTTCAG	GTTGTCACCGTAGTTAGCCATCAC
IL-6	CCAATCTGGGTTCAATCAGGAGAC	CAGCCTCGACATTTCCCTTATTGC
IL-10	AAGACGTAATGCCGAAGGCAGAGA	TGCTAAAGGCACTCTTCACCTCCT

3.3 Results

3.3.1 *Response of the U-937 macrophage cell line to FBS-containing hLCCA1 medium*

The activation of macrophages by secreted hCLCA1 was investigated using the U-937 macrophage cell line treated with conditioned FBS-containing medium from HEK293 cells heterologously expressing hCLCA1 or eGFP. Macrophage activation was demonstrated by expression of the pro-inflammatory cytokines IL-8, IL-6, IL-1β and TNF-α (Figure 3.1). The presence of secreted hCLCA1 in the medium was confirmed by western blot analysis (Figure 3.2). There was more cleaved hCLCA1 product than the hCLCA1 precursor present in both wild-type hCLCA1 lysate and medium, whereas, E157Q hydrolase-inactive hCLCA1 had only the precursor present. Densitometry analysis showed that there was no significant difference between the expression of wild-type hCLCA1 and E157Q mutant hCLCA1 in medium (Figure 3.2). In a log-scale dose test from 0.1 mg/mL to 10 mg/mL of total protein from the conditioned medium, the strongest response for these cytokines was obtained at 1 mg/mL (Figure 3.1). Concentrated pure medium and conditioned medium from HEK293 cells expressing the large protein TMEM16A (tested as an additional control) was found to cause no significant activation (Figure 3.1). At the optimized dose of 1 mg/mL, the expression of IL-8, IL-6, IL-1β and TNF-α in response to wild-type hCLCA1 and its hydrolase-inactive E157Q mutant was followed over 24 h (Figure 3.3); this test revealed no difference in the mRNA expression between E157Q and wild-type hCLCA1-activated macrophages. Generally, the longer the macrophages were incubated with hCLCA1, the higher the activation (Figure 3.3). Lipopolysaccharide (LPS), a potent endotoxin that activates through toll-like receptor 4, was used as a positive control and produced a similar time-dependent activation.

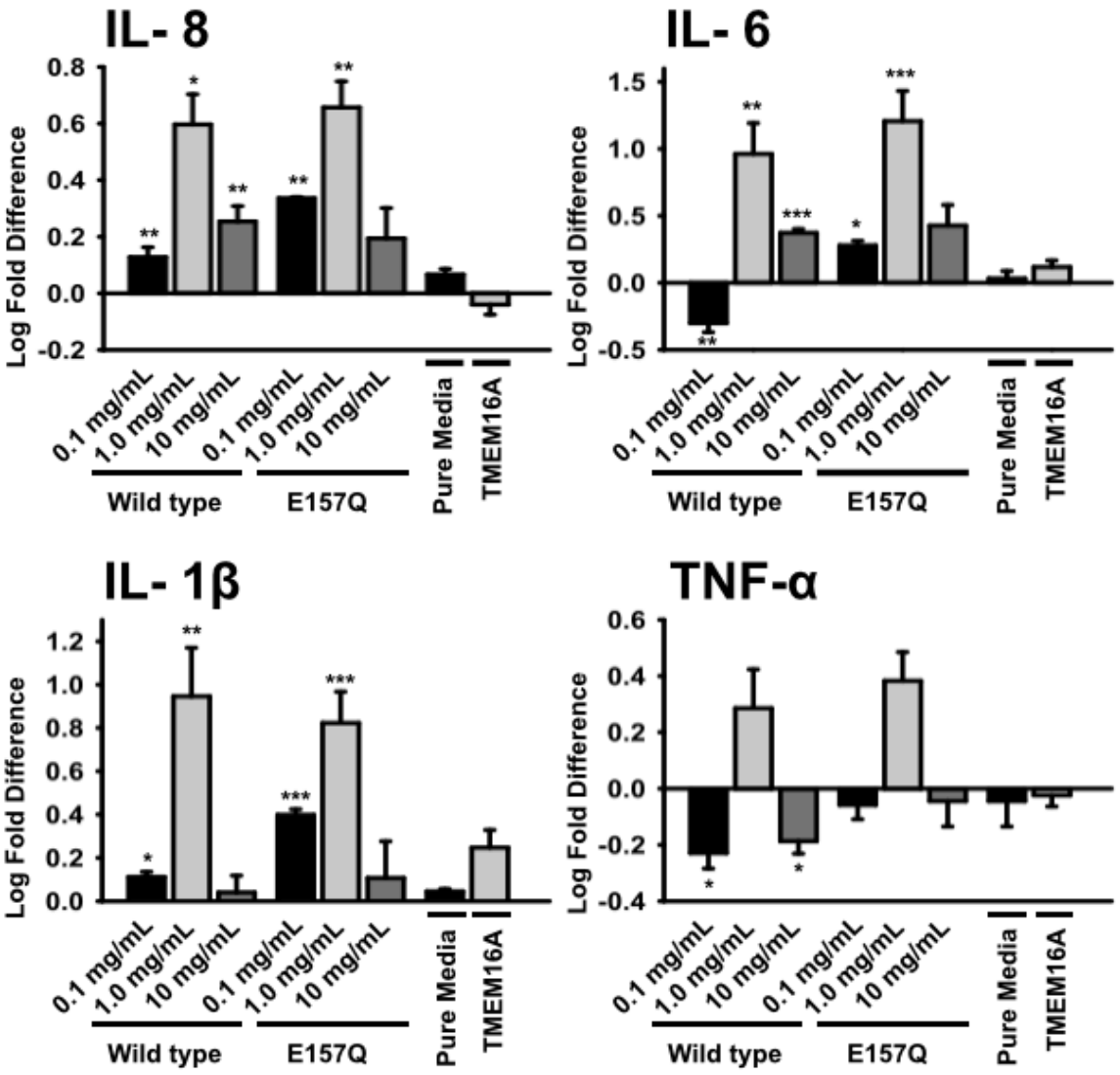

Figure 3.1. Conditioned FBS-containing hCLCA1 medium shows dose-dependent effect. Macrophages were activated for 24 h with 0.1 mg/mL, 1.0 mg/mL, or 10 mg/mL of FBS-containing stimulants (eGFP, wild-type hCLCA1, E157Q mutant hCLCA1, 1.0 mg/mL pure media, or 1.0 mg/mL TMEM16A). Dose-dependent cytokine mRNA expression by macrophages was quantified through RT-qPCR. The fold difference at each condition was compared against eGFP (the control) of the same concentration. Results were presented as the means of 3 samples ± SEM. Each sample was a result of an individual transfection paired with an eGFP transfection. Significant fold differences from corresponding control values (eGFP of the same concentration) are indicated by * ($p < 0.05$), ** ($p < 0.005$) or *** ($p < 0.001$).

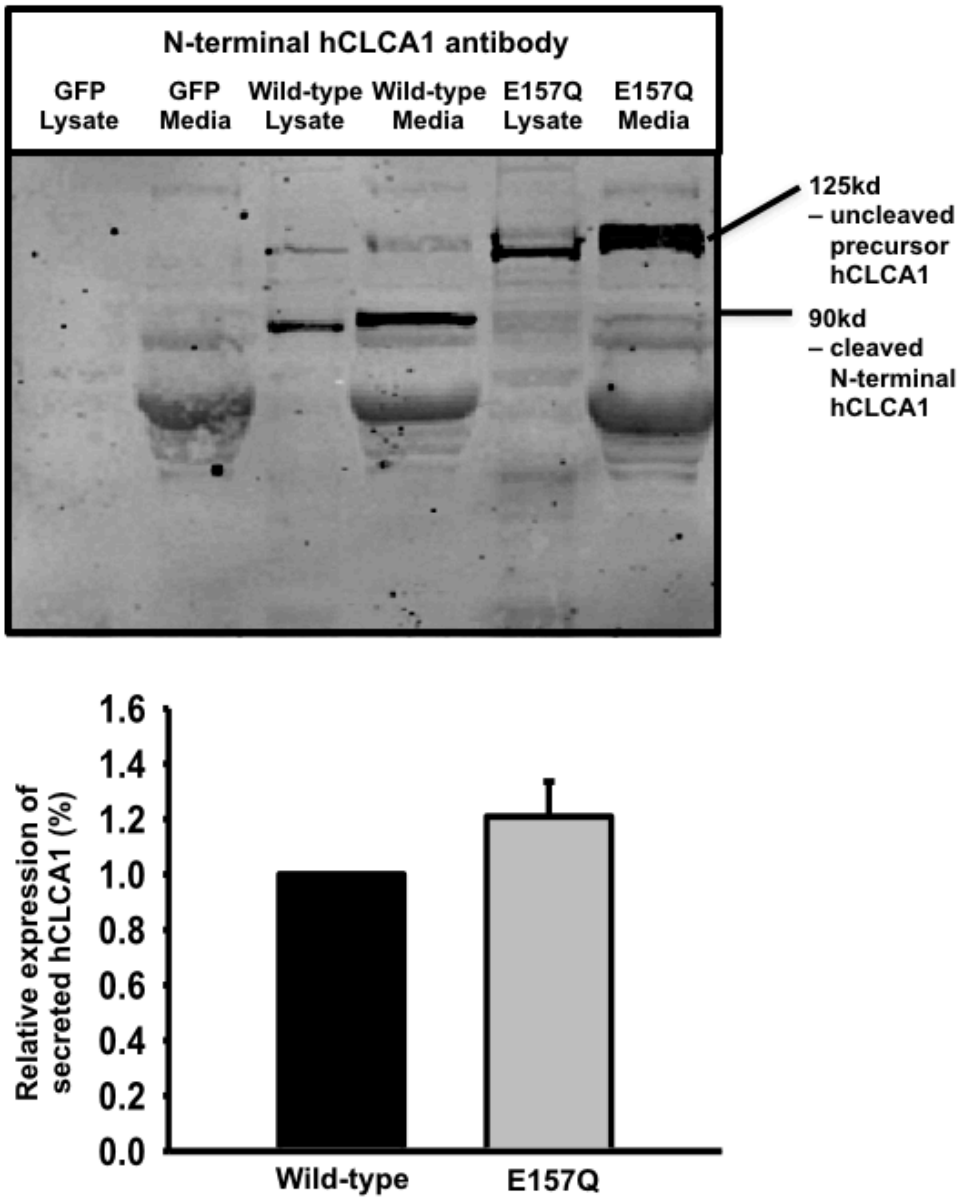

Figure 3.2. **Representative western blot image of eGFP- and hCLCA1-transfected HEK-293 cell lysates and media using hCLCA1 N-terminal antibody.** Both precursor and N-terminal hCLCA1 products were secreted into the extracellular space. However, there was a higher proportion of cleaved product than precursor in the wild-type hCLCA1 lysate and medium. Relative protein expressions of secreted wild-type and secreted E157Q mutant hCLCA1 were compared using densitometry (the secreted E157Q mutant hCLCA1 was normalized to the secreted wild-type hCLCA1 in each sample). No statistical significant difference was found between the protein levels of wild-type andE157Q mutant hCLCA1 in the medium. Wild-type hCLCA1 included 125 kDa uncleaved precursor and 90kDa cleaved N-terminal hCLCA1, while E157Q mutant hCLCA1 only included 125kDa uncleaved precursor hCLCA1. Results were presented as the means of 3 samples ± SEM.

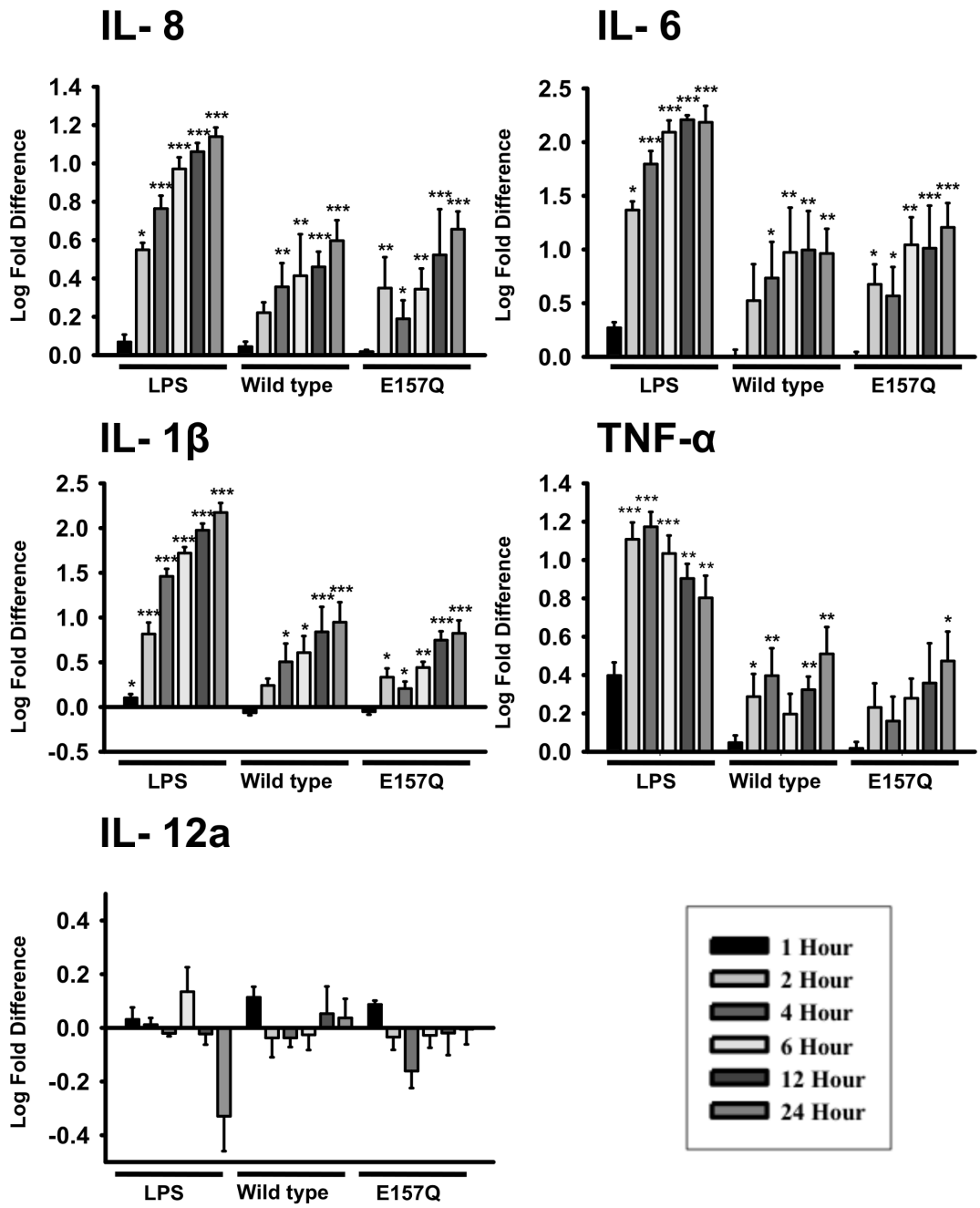

Figure 3.3. Conditioned FBS-containing hCLCA1 medium shows time-dependent effect. Macrophages were activated with 1.0 μg/mL LPS, 1.0 mg/mL FBS-containing wild-type hCLCA1, E157Q mutant hCLCA1 or eGFP medium. Time-dependent (1, 2, 4, 6, 12, 24 h) cytokine mRNA expression by macrophages was quantified through RT-qPCR. The fold difference at each condition was compared against eGFP (the control) of the same condition. Results were the means of 4 samples ± SEM. Each sample was a result of an individual transfection paired with an eGFP transfection. Significant fold differences from the corresponding control values (eGFP of the same concentration) are indicated by * ($p < 0.05$), ** ($p < 0.005$) or *** ($p < 0.001$).

3.3.2 *Dose response of the U-937 cell line to FBS-free conditioned hCLCA1 medium*

The macrophage growth medium for the dose response experiment was supplemented to the optimized concentration of 6% FBS, as determined by cytokine response (Figure 3.4). An appropriate FBS concentration was essential for macrophage growth and activation. The expression of hCLCA1 was confirmed by western blot (Figure 3.5A). Comparing between the FBS-containing medium and FBS-free medium, both western blot and coomassie gel (Figure 3.5B) staining showed that there was significantly less impurities in the FBS-free medium (Figure 3.5). The dose effect of conditioned FBS-free hCLCA1-containing medium on macrophage activation was carried out to eliminate any effects of FBS in the FBS-containing hCLCA1 medium (Figure 3.6). The concentration of total secreted protein in the medium used in Figure 3.1 was estimated to be between 50 and 200 μg/mL because we estimated that hCLCA1 only made up of ~1% of the total protein in the FBS-containing conditioned medium from coomassie stained gel. Therefore, concentrations of FBS-free hCLCA1 medium ranging from 3.3 μg/mL to 200 μg/mL were used for the macrophage activation dose response test, in which 66.7 μg/mL of FBS-free hCLCA1 medium was found to provide the maximum increase in the expression of IL-8, IL-6, and IL-1β; conversely, 200 μg/mL of FBS-free hCLCA1 medium caused a significant decrease in the anti-inflammatory cytokine IL-10 (Figure 3.6). The level of IL-12a never responded significantly and was generally just at the level of detection (Figure 3.6).

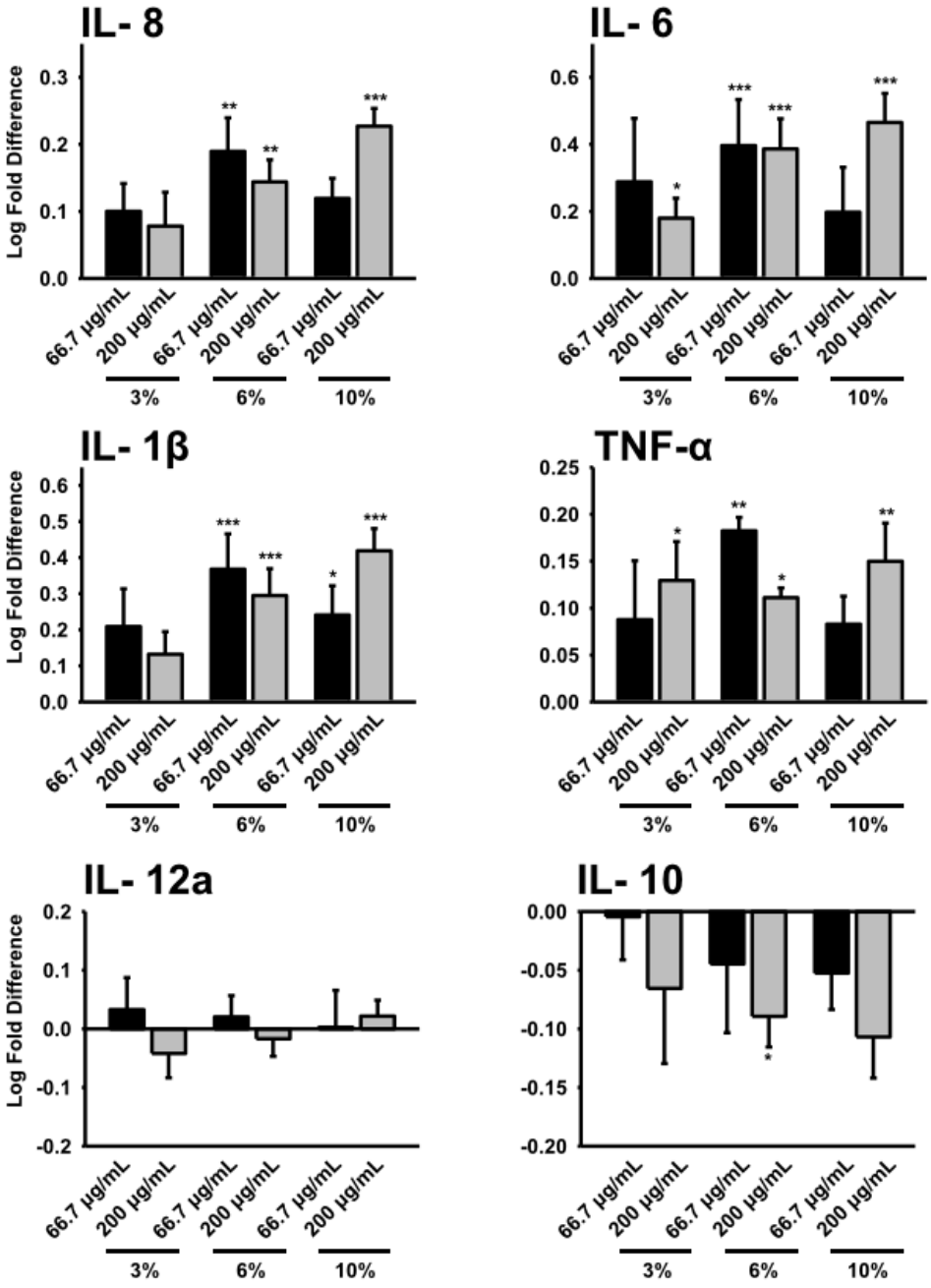

Figure 3.4. 6% FBS is determined to be the optimal FBS % in growth medium to activate macrophages. U-937 macrophage cells were activated for 24 h using 66.7 µg/mL or 200 µg/mL of FBS-free eGFP medium or FBS-free wild-type hCLCA1 medium with 3%, 6%, or 10% FBS growth medium. IL-8, IL-6, IL-1β, TNF-α, IL-12a and IL-10 were quantified by their mRNA expression using RT-qPCR. The fold difference at each concentration was compared against eGFP (the control) at the same concentration. Results were the means of 5 samples ± SEM. Each sample was a result of an individual transfection paired with an eGFP transfection. Significant fold differences from corresponding control values (eGFP of the same concentration) are indicated by * ($p < 0.05$), ** ($p < 0.005$) or *** ($p < 0.001$).

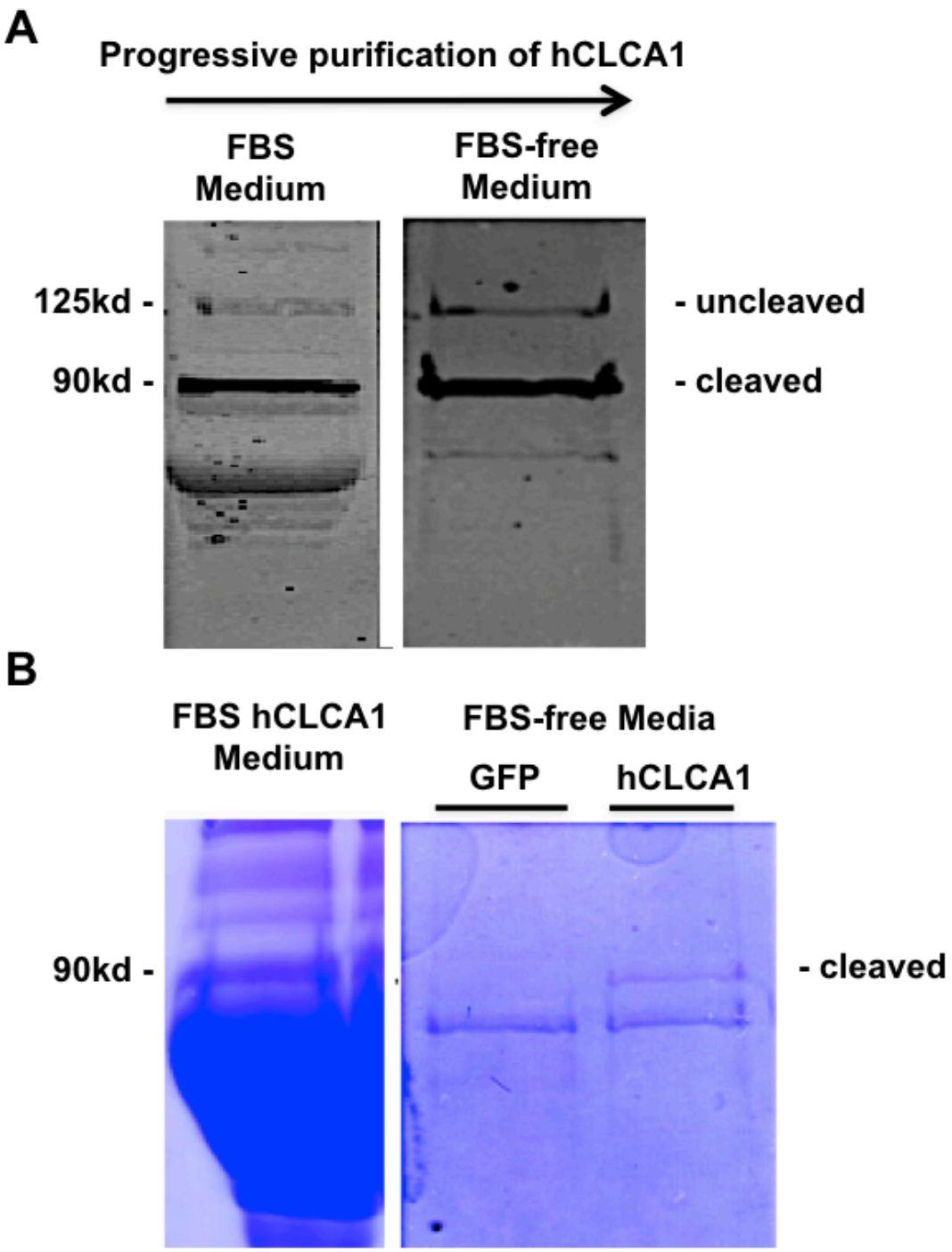

Figure 3.5. Representative western blot and coomassie gel showing FBS-containing and FBS-free conditioned media. (A) Western blot analysis probing against hCLCA1 N-terminal antibody was utilized in conjunction with **(B)** Coomassie gel staining to compare the total protein contents between FBS-containing hCLCA1 medium and FBS-free hCLCA1 medium. Both western blot and coomassie gel staining showed that hCLCA1 was one of the major secreted molecules, and there was less impurities in the FBS-free hCLCA1 medium without a major loss in hCLCA1 protein.

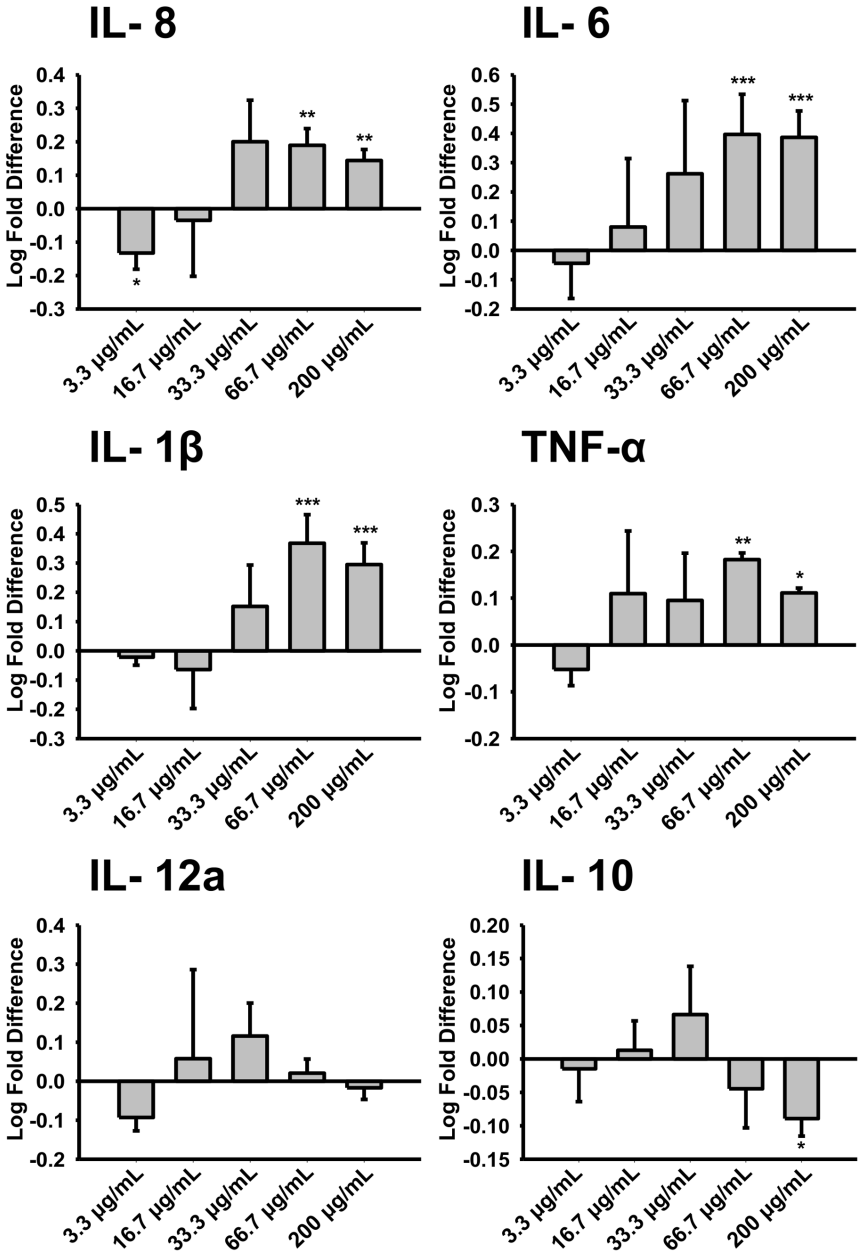

Figure 3.6. Conditioned FBS-free hCLCA1 medium shows a dose-dependent effect. U-937 macrophages were activated for 24 h using 3.3 µg/mL, 16.7 µg/mL, 33.3 µg/mL, 66.7 µg/mL, or 200 µg/mL of FBS-free wild-type hCLCA1 medium or FBS-free eGFP medium in 6% FBS growth medium. IL-8, IL-6, IL-1β, TNF-α, IL-12a and IL-10 were quantified by their mRNA expression using RT-qPCR. The fold difference at each concentration was compared against eGFP (the control) at the same concentration. Results were the means of 5 samples ± SEM. Each sample was a result of an individual transfection paired with an eGFP transfection. Significant fold differences from corresponding control values (eGFP of the same concentration) are indicated by * ($p < 0.05$), ** ($p < 0.005$) or *** ($p < 0.001$).

3.3.3 *Activation of primary porcine macrophage by hCLCA1*

To confirm that the effect of hCLCA1 was not specific to the U-937 cell line, isolated porcine alveolar macrophages were tested with FBS-free hCLCA1 or eGFP medium. Activation by hCLCA1 was observed in 20% FBS medium, a standard growth condition for isolated porcine macrophages (Figure 3.7). However, 66.7 μg/mL of FBS-free hCLCA1 medium activated only IL-1β; 200 μg/mL of FBS-free hCLCA1 medium was required to increase most of the pro-inflammatory cytokines (including TNF-α, IL-8, IL- 6 and IL-1β). At 1000 μg/mL FBS-free hCLCA1 medium, however, the pro-inflammatory response was reduced.

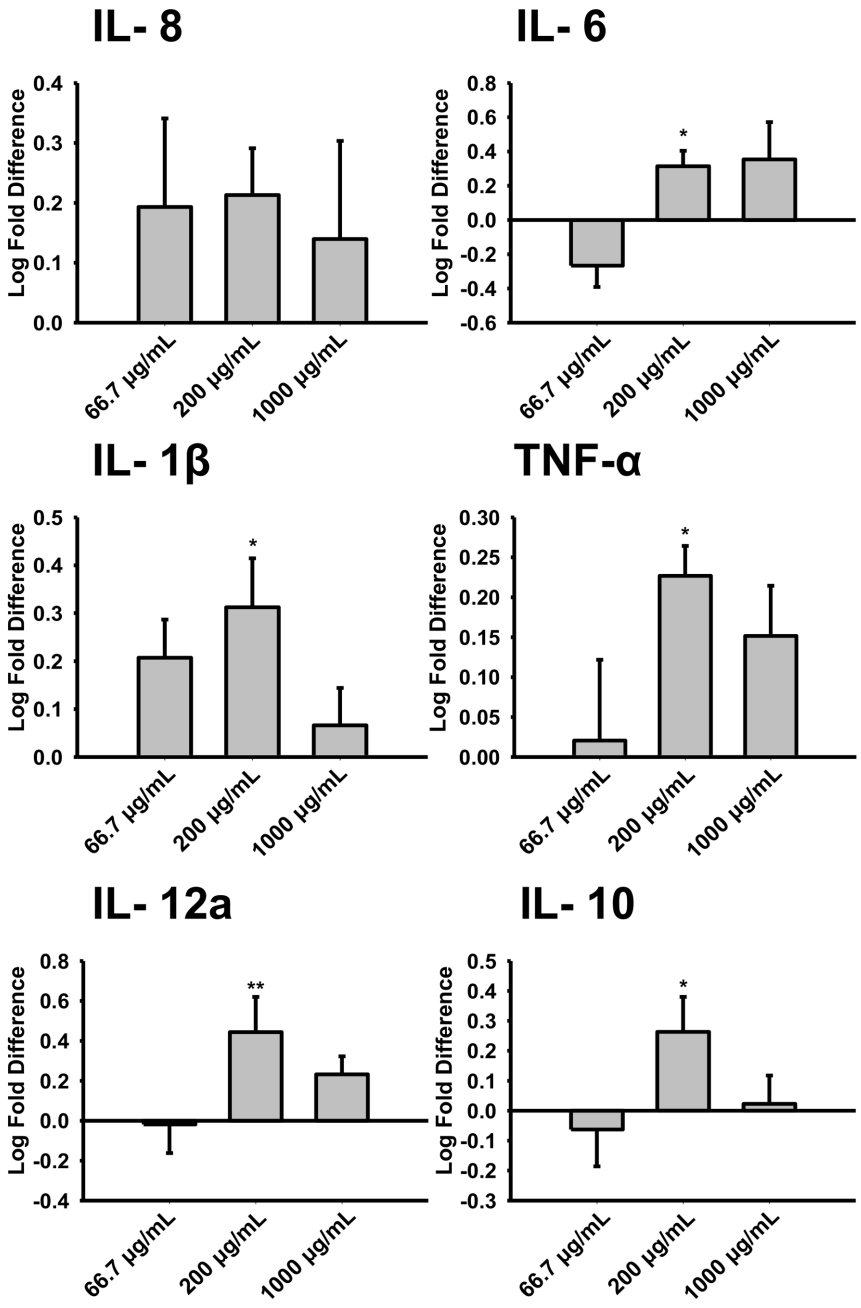

Figure 3.7. Activation of porcine alveolar macrophages with hCLCA1. Porcine alveolar macrophages were activated with different concentrations (66.7 µg/mL, 200 µg/mL or 1000 µg/mL) of FBS-free eGFP medium or FBS-free wild-type hCLCA1 medium. IL-8, IL-6, IL-1β, TNF-α, IL-12a and IL-10 were quantified by their mRNA expression using RT-qPCR. The fold difference at each concentration was compared against eGFP (the control) at the same concentration. Results were the means of 7 samples ± SEM. Each sample was a result of an individual transfection paired with an eGFP transfection. Significant fold differences from corresponding control values (eGFP of the same concentration) are indicated by * ($p < 0.05$), ** ($p < 0.005$) or *** ($p < 0.001$).

3.3.4 *Activation of the U-937 cell line by immuno-purified hCLCA1*

To demonstrate that hCLCA1 was acting on its own and not in conjunction with other components secreted by the HEK293 cell line, immuno-purified hCLCA1 from FBS-free medium was tested. The presence and purity of hCLCA1 was confirmed with western blot and silver stain (Figure 3.8A). Since the concentration of immuno-purified hCLCA1 was beyond the detection range of Bradford protein assay (> 1 μg/mL), we measured the concentration of hCLCA1 using a standard curve generated by a 2-fold dilution series of lysozyme on a silver stained SDS-PAGE gel (Figure 3.8B). Densitometry analysis showed that the concentration of immuno-purified hCLCA1 was 6.2 ± 0.3 pg/μL (Figure 3.8B). Therefore, the maximum hCLCA1 concentration achievable for macrophage activation was 93.3 pg/mL. Macrophages stimulated with 93.3 pg/mL of pure hCLCA1 activated similarly to that induced by the conditioned FBS-free medium after 48 h, although only IL-1β levels were enhanced after 24 h (Figure 3.9).

To demonstrate that higher concentration of pure hCLCA1 can elicit a stronger effect on macrophages, we optimized the immune-precipitation protocol to yield a higher hCLCA1 concentration. The improvement resulted in the concentration of immuno-purified hCLCA1 as determined through densitometry analysis on a silver stained SDS-PAGE gel to be 9.4 ± 0.3 pg/μL (Figure 3.10). This allowed macrophages to be activated with a maximum concentration of 141.7 pg/mL immuno-purified hCLCA1 for 48 h. Enhanced mRNA expression of pro-inflammatory cytokines was observed with increased hCLCA1 concentration (Figure 3.11A). For example, mRNA expression levels of IL-1β increased significantly from 0.178 log folds to 0.317 log folds ($p < 0.05$) with the increase in hCLCA1 concentration (Figure 3.9 and 3.11A).

The increase in cytokine mRNA expression mentioned above was translated into an elevated cytokine protein levels. Western blot and densitometry analysis showed that intracellular IL-1β protein levels increased by 2.38 ± 0.21 folds ($p < 0.001$) over the control (Figure 3.11B). However, neither LPS nor hCLCA1-activated macrophages produced detectable extracellular IL-1β protein in the medium according to western blot analysis

(data not shown). This suggests that IL-1β release is dependent on factors other than activation in this cell line. We then used a Bio-Plex suspension array system to measure the extracellular cytokine protein expression levels in the medium of control immuno-purified eGFP- and hCLCA1-activated macrophage. Significant fold increase over control protein levels of IL-6 and IL-8 was observed, while IL-4, IL-10, GM-CSF, IFN-γ, and TNF-α protein levels did not change significantly; and IL-2 was beyond the detection limit (Figure 3.11C).

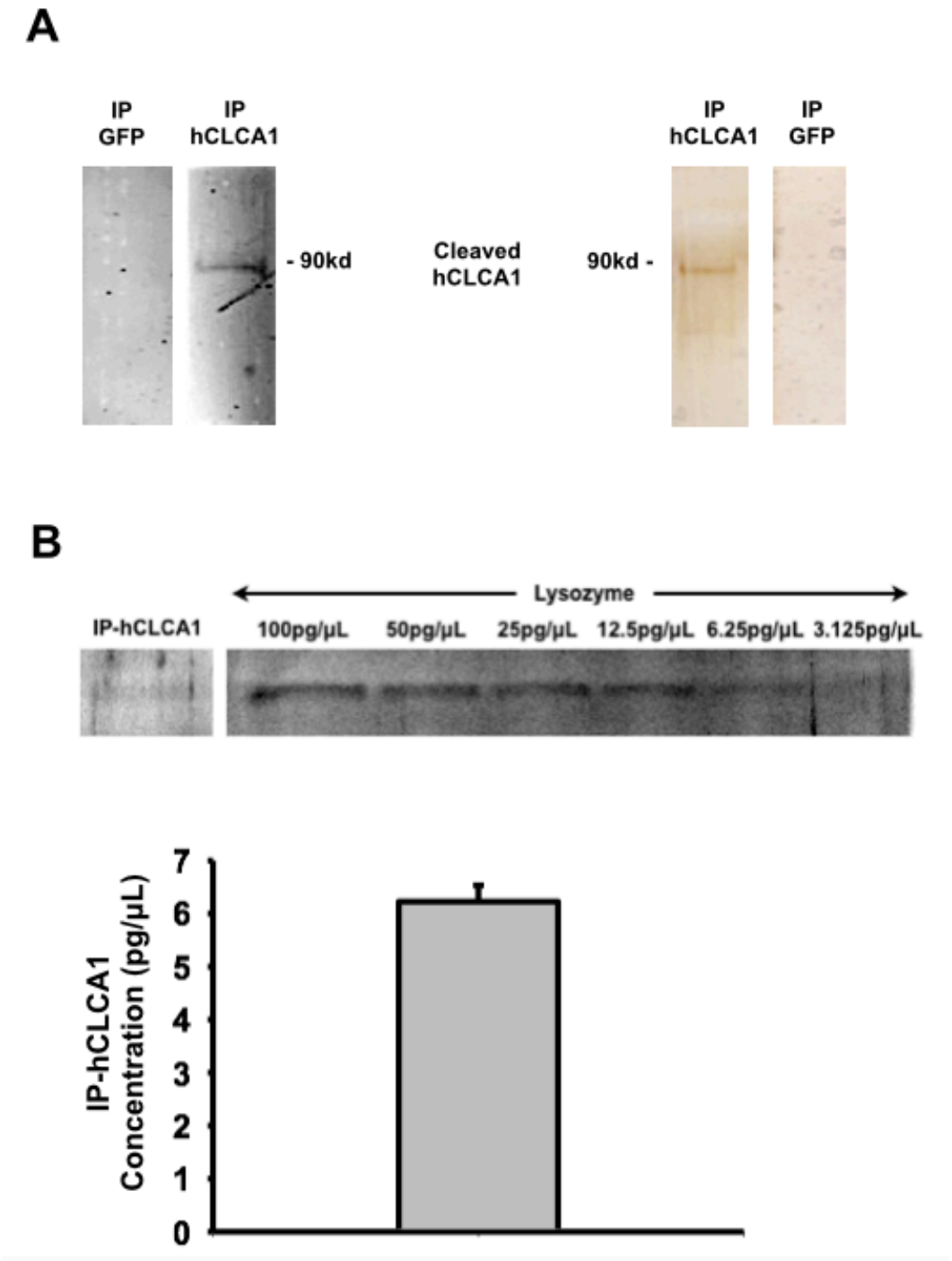

Figure 3.8. Representative western blot and silver stained gel on immuno-purified hCLCA1. (A) Western blot analysis of immuno-purified hCLCA1 and eGFP using hCLCA1 N-terminal antibody; and silver stained acrylamide gel containing immuno-purified hCLCA1 and eGFP. (**B)** Using a standard curve generated from a 2-fold dilution series of lysozyme on a silver stained SDS-PAGE gel, immuno-purified hCLCA1 was determined to be 6.225 ± 0.307 pg/µL. Result was presented as the mean of 3 samples ± SEM.

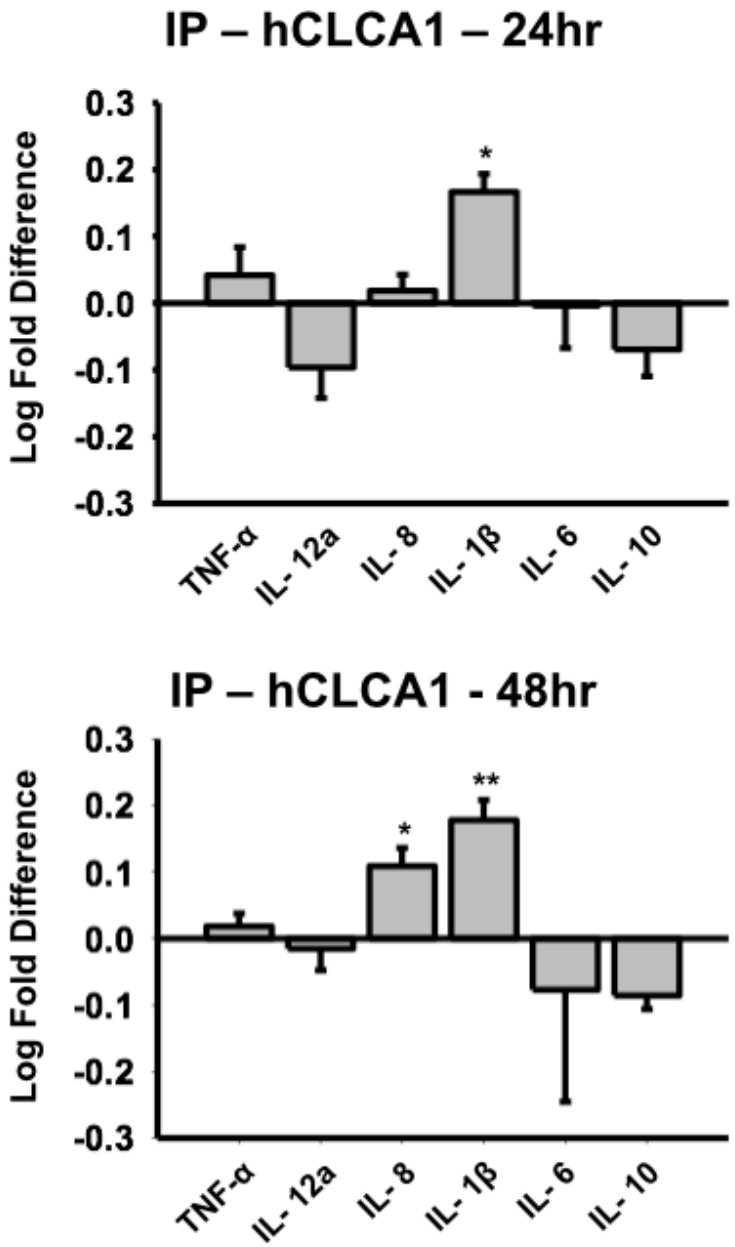

Figure 3.9. Activation of macrophages with immuno-purified hCLCA1. The mRNA expression of cytokines in macrophages stimulated with immuno-purified hCLCA1 for 24 h and 48 h were quantified using RT-qPCR. The fold difference was calculated against the corresponding control (immunoprecipitation of eGFP using hCLCA1-N14 antibody). Results for and were presented as the means of 10 samples ± SEM. Each sample was a result of an individual transfection paired with an eGFP transfection. Significant fold differences from corresponding control values (immuno-purified eGFP) are indicated by * ($p < 0.05$), ** ($p < 0.005$) and *** ($p < 0.001$).

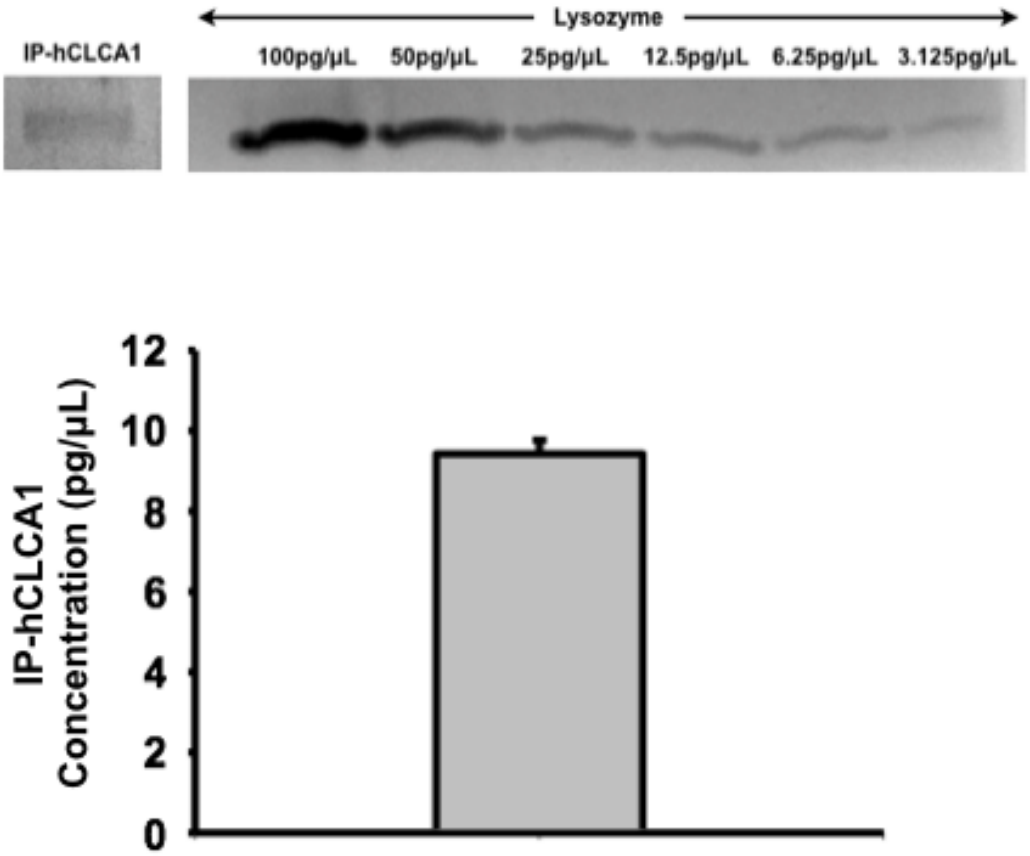

Figure 3.10. Representative silver stained SDS-PAGE gel showing immuno-purified hCLCA1 with optimized protocol and a 2-fold dilution series of lysozyme. Densitometry analysis using lysozyme standard curve demonstrated a higher concentration of pure hCLCA1 (9.425 ± 0.335 pg/µL) was immunoprecipitated with an optimized protocol. The optimized protocol yield a ~50% increase in protein amount compared to the original protocol. Result was presented as the mean of 3 samples ± SEM.

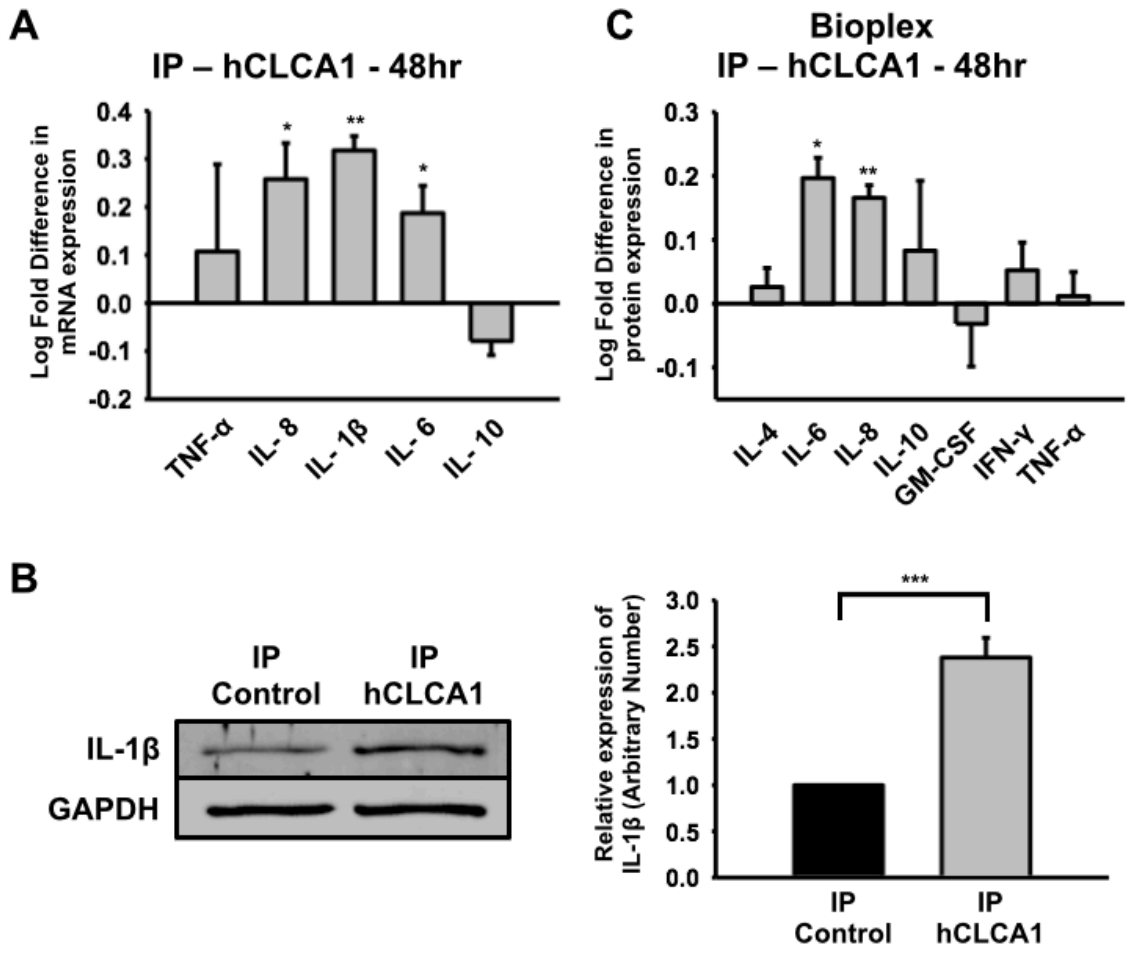

Figure 3.11. Enhanced macrophage activation with higher concentration of immuno-purified hCLCA1. (A) The mRNA expression of cytokines in macrophages stimulated with 141.7 pg/mL of hCLCA1 for 48 h was quantified using RT-qPCR. The fold difference was calculated against the corresponding control (immunoprecipitation of eGFP using hCLCA1-N14 antibody). Results were presented as the means of 4 samples ± SEM. **(B)** Representative Western blots showing intracellular IL-1β and GAPDH levels in immuno-purified eGFP or hCLCA1-stimulated macrophages. GAPDH was used as a loading control for densitometry analysis. Immuno-purified hCLCA1-stimulated macrophages had a 2.38 ± 0.21 folds increase in IL-1β protein levels over immuno-purified eGFP-stimulated macrophages (the hCLCA1-induced IL-1β was normalized to the eGFP-induced IL-1β in each sample). Results were presented as the means of 7 samples ± SEM. **(C)** Secreted cytokine protein expression in macrophages stimulated with a higher concentration of immuno-purified hCLCA1 was analyzed using Bio-plex Suspension Array System. The fold difference of each sample was compared against the corresponding control. Results were the means of 3 samples ± SEM. Significant fold differences from corresponding control values are indicated by * ($p < 0.05$), ** ($p < 0.005$) and *** ($p < 0.001$).

3.4 Discussion

The results demonstrate that secreted hCLCA1 is able to function as a signaling molecule by activating both cell-line and primary porcine alveolar macrophages in a dose- and time-dependent manner. Confirmation of the function of hCLCA1 as a signaling ligand for airway macrophages can begin to explain its pleiotropic effects[294].

3.4.1 *Type of response*

The signaling function was initially demonstrated using conditioned FBS-containing medium from HEK293 cells heterologously expressing hCLCA1. At an optimal dose of 1 mg/mL, the conditioned FBS-containing medium activated macrophages over time similarly to the effect of LPS (Figure 3.3). To stimulate macrophages without drastically changing the ionic and nutrient composition of the medium, the protein was concentrated out of the conditioned medium. However, this procedure also concentrated the FBS, which could have been deleterious to the macrophages at concentrations of 10 mg/mL, reducing the pro-inflammatory response (Figure 3.1). Alternatively, the high concentrations of FBS and hCLCA1 together could turn off the pro-inflammatory response.

Interestingly, activating the macrophages with FBS-free hCLCA1 at higher concentrations of FBS (10%) in the growth media resulted in only a slight to moderate decrease in the pro-inflammatory response (Figure 3.4). This decrease was not as significant as that of the 10 mg/mL (concentrated) hCLCA1 in FBS-containing medium (Figure 3.1). This difference suggests that a high concentration of hCLCA1 itself may actually begin to dampen the pro-inflammatory response, a trend that was seen with FBS-free hCLCA1-conditioned medium when applied to a macrophage cell line (Figure 3.6). Evidence for higher concentrations of hCLCA1 having a reduced pro-inflammatory response was salient in the porcine alveolar macrophage experiment, where the strong pro-inflammatory response induced by low concentrations of FBS-free hCLCA1-containing medium was lost at higher concentrations (Figure 3.7).

Together, these results demonstrate that hCLCA1-containing medium has the capacity to induce a pro-inflammatory response that is dependent on its concentration, and the effect of activation is not cell-line dependent. In addition, these results also illustrated hCLCA1's ability to activate macrophages within a mixed milieu of macromolecules. A strong pro-inflammatory response was observed when macrophages were activated with 1 mg/mL of FBS-containing hCLCA1 conditioned medium (Figure 3.1). The FBS-containing hCLCA1 conditioned medium is comprised of a large number of immuno-reactive factors such as cytokines or growth factors. In pathophysiological settings, hCLCA1 would also have to function in such an environment of macromolecules and immuno-reactive factors presented in the BAL fluid of inflamed airways[307].

3.4.2 *Macrophage activation by pure hCLCA1*

To confirm that macrophage activation is not dependent on constituents that might potentially be induced and secreted by hCLCA1 in HEK293 cells, we immunoprecipitated hCLCA1 from FBS-free hCLCA1-containing medium. In the initial experiment in which 93.3 pg/mL of hCLCA1 was used to activate macrophages, the response was limited to IL-1β in the first 24 h. Up to 48 h of macrophage activation was required for IL-8 to increase significantly as well (Figure 3.9). It should be noted that 93.3 pg/mL of hCLCA1 is lower than the physiological concentration secreted from human airway epithelial cells[41], thus a weak response was expected. However, increasing the dose to 141.7 pg/mL of hCLCA1 elicited a stronger cytokine mRNA expression in macrophages at 48 h (Figure 3.11A), demonstrating pure hCLCA1's ability to function in a dose dependent manner. This increase in cytokine mRNA expression was sufficient to increase cytokine protein expression, further supporting the physiological relevance of these findings (Figure 3.11B and 3.11C). In fact, the concentration of hCLCA1 employed in this study is similar to physiological concentrations of signaling molecules found in inflamed airway, as many cytokines are expressed in the pg/mL to ng/mL range in bronchoalveolar lavage (BAL) fluid in asthmatic patients[307-309].

3.4.3 *Potential activation mechanisms*

Taken together, these findings indicate that airway macrophage activation is an intrinsic property of hCLCA1. hCLCA1 can elicit a concentration-dependent macrophage activation. Generally, activation of pro-inflammatory response has a receptor-driven signal transduction mechanism and is dependent on ion-channel activation at the cell surface[298, 300].

The protein hCLCA1 could also activate macrophages by ion-channel modulation. The ability of CLCA proteins to modulate multiple ion-channel types has been well-documented[59, 60, 310]. These proteins increase single-channel conductance and directly interact with channel subunits[36, 311]. However, inhibiting the hydrolase domain of CLCA proteins inhibited their capability to modulate calcium-activated chloride channels (CaCCs)[70]. Our findings suggest that since the hydrolase-inactive mutant could activate macrophages, then the CaCC-modulating ability of hCLCA1 is not involved in macrophage activation. However, such results would not preclude hCLCA1 being the modulator of other channels that are independent of its hydrolase domain. HCLCA1's Von Willebrand Factor-A (VWA) domain could for example act as a ligand to modulate ion channels. Such precedents exist as the $\alpha_2\delta$ subunit of the voltage gated calcium channel modulates its function by binding to an extracellular region of the channel pore subunit via its VWA domain and modifies its function[74].

Alternatively, hCLCA1 macrophage activation may occur through a signal transduction mechanism driven by a receptor ligand. Precedents exist, as some CLCA homologs contain novel integrin binding motifs[49, 312]. However, the orthologs expressed in airway cells, including hCLCA1, do not possess this binding motif. Another domain that could potentially cause macrophage activation is the FN3 domain in the C-terminus of the hCLCA1 protein. This FN3 domain in fibronectin has been shown to induce cytokine expression in lung fibroblasts[80]. However, increasing the concentration of the FN3 domain in the medium by mutating the hydrolase domain did not increase macrophage activation (Figures 3.1).

3.4.4 *Pathophysiological implications*

The signaling ability illustrated by our findings begins to explain how CLCA has such a pleiotropic effect on airway inflammation[294]. This effect was first demonstrated using airway adenoviral gene transfer expressing CLCA in BALB/c mice, in which the artificial expression drove and exacerbated mucus production, goblet cell metaplasia, eosinophil infiltration and airway hyper-responsiveness in an allergic asthmatic model[43]. However, subsequent studies in C57BL/6 and 129v mouse strain backgrounds produced both conflicting and corroborating results[54, 99, 294, 313]. These conflicting studies could be explained if CLCA was functioning as a signaling protein which modulates a central mediator of the immune response (such as macrophages), which have a pleiotropic effect on lung inflammation. It is known that lung macrophages differ significantly between mouse strains, and such variations could explain the differences seen in the CLCA knockout models[43, 99, 294, 313, 314].

3.5 Conclusions

We have described the novel ability of secreted hCLCA1 to function as a signaling molecule that can activate airway macrophages. Such ability likely has a profound impact on the immune response in the airways, where the expression of this gene is massively up-regulated during inflammation[14]. Airway macrophages are pivotal regulators of the inflammatory response, and the ability of hCLCA1 to activate them could explain the pleotropic effect seen in airway inflammation models (where hCLCA1 is either over-expressed or knocked out). The cytokine responses of the macrophages would then modify the inflammatory response, mucus secretion, airway hyper-responsiveness and epithelial ion-channel function (Figure 5.1). Cytokines are known to modulate these processes[315, 316]. Studies to identify the receptor and hCLCA1 domain containing the corresponding ligand are underway but are beyond the scope of this paper. The VWA domain could be one potential candidate, where the same domain in α2δ subunit binds to voltage-gated calcium channel[74]. The receptor and the ligand domain of hCLCA1 should be of therapeutic interest, as blocking either could have beneficial effects against airway inflammation. Furthermore, these findings open a new area of investigation into the function of CLCA proteins.

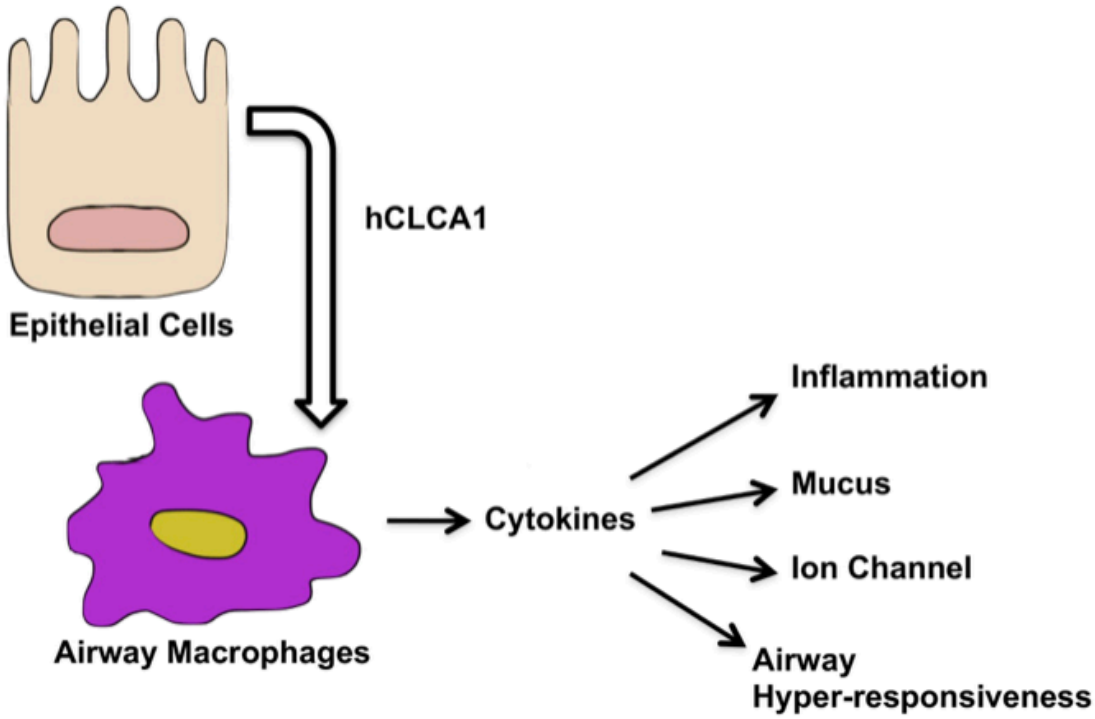

Figure 3.12. Schematic model of the effects of hCLCA1 on airway macrophages. We hypothesized that hCLCA1 secreted from inflamed epithelial cells induces airway macrophages to secrete pro-inflammatory cytokines, and the cytokines will modulate different physiological functions including inflammation, mucus expression, ion-channel activity, and airway hyper-responsiveness.

STUDY 2 – VON WILLEBRAND FACTOR TYPE A DOMAIN OF HCLCA1 IS RESPONSIBLE FOR MACROPHAGE ACTIVATION

The human hCLCA1 gene is a member of *CLCA* gene family that has a well-documented role in inflammatory airway diseases. It has a pleiotropic effect on different physiological functions including modulation of ion-channel functions and induction of mucus production. Previously, we demonstrated that secreted hCLCA1 plays a role in regulating the innate immune response by activating airway macrophages. However, the mechanism for this regulation remains unclear. In this present study, we expressed recombinant proteins containing different hCLCA1 domains to determine the specific hCLCA1 domain(s) responsible for activation and mechanisms that are involved in modulating immune response in macrophage. We showed that the von Willebrand factor type A (VWA) domain was responsible for activating macrophages. Macrophages treated with VWA domain exhibited increases in both IL-1β mRNA and protein expressions which correlate with the activation of MAPKs and NF-κB pathways.

4.1 Introduction

The human hCLCA1 is a member of *CLCA* (*CL* stands for chloride-channel modulating and *CA* for calcium-activated) family with a well-established role in inflammatory airway diseases such as asthma, cystic fibrosis and chronic obstructive pulmonary disease[25, 96, 163, 186]. Studies have reported that hCLCA1 is induced in inflamed airway epithelial cells, and this induced expression often exceeds that of most other inflammatory mediators[14, 293]. The gene products have a pleiotropic effect, generating secreted and membrane-associated proteins that increase mucus production, airway responsiveness, and increase calcium activated chloride channel conductance[14, 21, 36, 41, 43, 85, 92, 96, 163, 293-295]. Although hCLCA1 was originally identified as the pore subunit of the calcium activated chloride channels, we and others concluded that it is a chloride channel accessory protein instead[1, 4, 36, 59, 60]. However, how this accessory protein is involved in many different cellular functions remains unknown. Recently, we have identified the novel role of hCLCA1 as a signaling molecule[45], suggesting a possible mechanism to its pleiotropic effect. A recent study demonstrated that secreted hCLCA1 increases the surface protein expression level of TMEM16A[37], further supporting our proposed role of hCLCA1 as a signaling molecule to modulate different physiological functions.

In our previous study, we have demonstrated hCLCA1 acts as a signaling molecule and modulates the innate immune response in macrophages[45]. A recent study also demonstrated that mCLCA3, the murine ortholog of hCLCA1, modulates leukocyte recruitment via IL-17 and CXCL-1 in bacterial pneumonia[100], which is in line with our newly identified function of hCLCA1 as a modulator in immune response. However, the mechanism regarding this modulation remains unclear. In this study, we expressed proteins containing different hCLCA1 domains to determine the specific hCLCA1 domain(s) and mechanisms that are involved in modulating the innate immune response in macrophage. From our results, we found that von Willebrand factor type A (VWA) domain of hCLCA1 is responsible for the up-regulation of both mRNA and protein expression of IL-1β. We also found that NF-κB and MAPK signaling pathways were activated when exposed to VWA domain, suggesting a possible mechanism of how hCLCA1's VWA domain activated macrophages.

4.2 Material and Methods

4.2.1 *Cell culture*

Human monocytes (U-937 cell line; CRL1593.2; ATCC) were grown in RPMI-1640 medium (SH3025502; Thermo Scientific) supplemented with 10% heat inactivated Fetal bovine serum (FBS; 16000-044; Life Technologies) and 1% penicillin-streptomycin (pen-strep; 15140-122; Life Technologies) at 37 °C in a humidified atmosphere with 5% CO_2.

4.2.2 *Plasmid construct*

The DNA of different hCLCA1 domains was amplified using polymerase chain reaction (PCR) with Phusion high-fidelity DNA polymerase (F531S; Thermo Scientific) according to manufacturers' protocols (Table 4.1). Hydrolase domain (N1-C273), von Willebrand Factor Type A (VWA) domain (N240-C544), Hydrolase-VWA domain (N1-C544), and Fibronectin type III (FN3) domain (N643-C915) were cloned into a modified pET28 HMT vector (containing in sequence, a hexahistidine-tag (His_6-tag), maltose binding protein (MBP), and a cleavage site for the tobacco Etch Virus protease (TEV) (Gift of Dr. F. Van Petegem)) using a ligation independent cloning method[317]. Briefly, pET28 HMT vector DNA was treated with T4 DNA polymerase reaction (10 µL DNA (1-2 µg), 2 µL NEBuffers 2.1, 0.5 µL 100 mM deoxyguanosine triphosphate (dGTP; N0446S; New England BioLabs), 1 µL 100mM dithiothreitol (DTT; BP172-5; Fisher Scientific), 0.4 µL T4 DNA polymerase (M0203L; New England BioLabs), and 6.1 µL H_2O). hCLCA1 domains DNA was also treated with T4 DNA polymerase reaction, however, deoxycytidine triphosphate (dCTP; N0446S; New England BioLabs) was used instead of dGTP. Both vector and inserts DNA were incubated for 40 min at room temperature and 20 min at 75 °C for heat inactivation, then incubated for 10 min at room temperature for the annealing process. The ligation reaction products were transformed into *Escherichia coli (E. coli)* DH5α strain competent cells (18258-012; Life Technologies) for DNA amplification.

For the transformation process, 1-2 µL of ligation reaction products were added into 100 µL of *E. coli* DH5α competent cells and incubated for 30 min on ice followed by 1 min heat shock at 42 °C. Next, 1 mL of Luria-Bertani (LB) broth (10 g/L NaCl, 5 g/L yeast extract (BP1422500; Fisher Scientific), and 10 g/L tryptone (BP1421500; Fisher Scientific)) was added to the cells and allowed 1 h incubation in a shaking incubator at 37 °C. The cells were spun down at 3000*g* for 1 min, and the cell pellets were plated on LB-agar plate (10 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone, and 15 g/L agar (214010; BD Biosciences) supplemented with 50 µg/mL kanamycin for 16-18 h at 37 °C. Then a single bacterial colony was picked and grown in 5 mL LB broth supplemented with kanamycin for 16-18 h at 37 °C in a shaking incubator, and the plasmid DNA was purified using the QIAprep Spin Miniprep Kit (27106; Qiagen) according to manufacturers' protocols. The purified plasmid DNA was sent for DNA sequencing (Macrogen Inc.) to confirm the presence of hCLCA1 domain sequence in pET28 HMT vectors (Table 4.2).

4.2.3 *Protein expression, purification, endotoxin removal and concentration determination*

For protein expression, the plasmid constructs were first transformed into *E. coli* strain Rosetta (DE3) competent cells (70954; Novagen) and plated on LB-agar plates supplemented with 50 µg/mL kanamycin and 35 µg/mL chloramphenicol. The transformed cells were grown in 2YT broth (5 g/L NaCl, 10 g/L yeast extract, 16 g/L tryptone) at 37 °C in a shaking incubator. Cultures were grown in 2YT broth to an OD_{600} ~ 0.3 at 37 °C, and the temperature was lowered to 18 °C. Once the cultures reached an OD_{600} ~0.6-0.7, the cells were induced with 0.2 mM isopropyl-β-D-thiogalactoside (IPTG; BP16201; Fisher Scientific) and allowed to grow for a total of 18-22 h.

The cultures were centrifuged for 20 min at 5000*g*, and the cell pellets were lysed by sonication after addition of 5 mL glycerol, 40 mL Buffer A (250 mM KCl, 10 mM HEPES pH 7.4) supplemented with 25 µg/mL DNase I (D5025; Sigma-Aldrich), 25 µg/mL lysozyme, 1mM phenylmethanesulfonyl fluoride (PMSF; P7626; Sigma-Aldrich) and 10 mM β-mercaptoethanol (BME; M6250; Sigma-Aldrich). The lysate was centrifuged for 30 min at

35,000*g* and a clarified supernatant was loaded onto BioLogic DuoFlow QuadTec 40 System (760-4137; BioRad) for protein purification.

The proteins were first purified using a Profinity IMAC Ni-charged resin column (156-0131; BioRad). The proteins were washed with 10 column volumes (CVs) of Buffer A followed by 5 CV of 2% Buffer B (250 mM KCl, 500 mM imidazole (O3196500; Fisher Scientific) and 5 CV of 4% Buffer B. The captured proteins were then eluted by a gradient of 4%-60% Buffer B (up to 300 mM imidazole). The protein elusions were diluted with an equal volume of buffer A and further purified using an amylose column (E8021L; New England BioLabs). The captured proteins were washed with 10 CV of Buffer A and eluted with 5 CV of Buffer C (250 mM KCl, 10 mM HEPES pH 7.4, and 10 mM Maltose). The eluted proteins were dialyzed (211528; Fisher Scientific) against Buffer D (10 mM KCl and 20 mM HEPES pH 7.4) at 4 °C overnight and were loaded onto a Resource Q anion exchange column (17-1179-01; GE Healthcare Life Sciences). The proteins were washed with 4 CV of Buffer D and eluted with a gradient of 0% - 100% Buffer E (1 M KCl and 20 mM HEPES pH 7.4). The proteins were concentrated with Amicon ultra-15 centrifugal filter units (UFC903008; EMD Millipore) and polished by gel-filtration chromatography on a Hiload 16/60 Superdex 200 (17-1071-01; GE Healthcare Life Sciences) by running 1 CV of Buffer A through the column (Table 4.3).

The eluted proteins were boiled in 2x denaturing buffer (20% glycerol, 4% SDS, 125mM Tris pH 6.8, 0.3mM bromophenol blue) and 10% BME, and they were analyzed by 12% SDS-PAGE. The SDS-PAGE gel was stained with coomassie blue stain (staining – 45% methanol, 10% glacial acetic acid, 45% water, 3 g/L Coomassie Brilliant Blue R250; destaining – 20% methanol, 10% glacial acetic acid, 70% water). After confirming the purity of the eluted proteins, they were concentrated to a final volume of ~1.5 mL, and were subjected to endotoxin removal assay using Triton-X114 phase separation technique. The purified proteins were treated with 1% triton-X114, incubated for 20 min at 4 °C, 10 min at 37 °C, and centrifugation for 10 min at 20,000*g*. This process was repeated 3x to completely remove endotoxin residuals from the proteins. The endotoxin level was validated using GenScript ToxinSensor chromogenic LAL endotoxin assay kit (L00350; GenScript) to ensure

the endotoxin level is below 0.01 EU/mL. The protein concentration of the purified proteins was determined using Pierce BCA protein assay kit (23225; Thermo Scientific) according to the manufacturers' protocols.

4.2.4 *Monocyte differentiation and activation*

Monocyte cells were seeded in each well (1.3×10^6 to 1.5×10^6 cells/well) in a 6-well plate and differentiated into macrophages with 0.1 nM phorbol-12-myristate-13-acetate (PMA; P8139; Sigma-Aldrich) in supplemented FBS-free RPMI-1640 medium for 18 h. The cells were washed 2 times with FBS-free RPMI-1640 medium and incubated in supplemented RPMI-1640 medium containing 6% FBS. For the IL-1β experiment, 1 μg/mL or 5 μg/mL of different purified hCLCA1 domain proteins were added to macrophages for 48 h. For the cell signaling experiment, 5 μg of HMT or VWA proteins were added to macrophages for 2, 4, 6, or 12 h.

4.2.5 *RNA isolation and real-time quantitative PCR*

RNA was extracted using TRIzol reagent (15596018; Life Technologies) according to manufacturers' protocols. The collected RNA was analyzed with a GoTaq 2-Step RT-qPCR system (A6010; Promega) and Mx3005P real-time PCR machine (401514; Agilent). cDNA of each sample was measured in duplicates in Mx3005P real-time qPCR machine, and the average C_T (cycle threshold) value was used to calculate the fold difference of each gene. Primers were designed for GAPDH, TNF-α, IL-12a, IL-8, IL-1β, IL-6 and IL-10 (Table 4.4).

4.2.6 *Efficiency and fold difference calculations*

Dilution series from 1×10^0-fold to 1×10^{-5}-fold of cDNA were used to determine the primer efficiency. The C_T value obtained in each dilution was used to generate a linear plot of C_T vs. log copies. The efficiency of the primer set was determined with the equation $Eff = 10^{(-1/\text{slope})}$. The fold difference between hCLCA1's domains-activated and unstimulated

samples was determined using an efficiency-corrected calculation with unstimulated macrophage serving as control and GAPDH serving as the reference gene[304]:

$$ratio = \left(Eff_{\text{target}}\right)^{\Delta C_{\text{T, target}}\left(\text{Mean control} - \text{Mean sample}\right)} / \left(Eff_{\text{ref}}\right)^{\Delta C_{\text{T, ref}}\left(\text{Mean control} - \text{Mean sample}\right)}$$

4.2.7 *SDS-PAGE and western blot Analysis*

For IL-1β experiments, cell lysates were collected using M-PER mammalian protein extraction reagent (78503; Thermo Scientific) with the addition of Halt protease and phosphatase inhibitor cocktail (78440; Thermo Scientific). Cell lysates were resolved by 12% SDS-PAGE and electroblotted onto PVDF membrane. Membranes were blocked with Amresco rapidblock blocking solution (M325; Amresco) for 1 h at room temperature. The membranes were subsequently probed overnight at 4 °C with primary antibodies in rapidblock blocking solution. The membranes were then incubated 1 h at room temperature with secondary antibodies in rapidblock blocking solution. The primary antibodies used were GAPDH (MAB374; EMD Millipore) and IL-1β (H-153; sc-7884; Santa Cruz), and the secondary antibodies used were DyLight 488 conjugate Goat anti-Rabbit IgG antibody (35552; Thermo Scientific) and ECL Plex Goat anti-Mouse IgG-Cy5 antibody (PA45009; Amersham Biosciences). Proteins were detected and analyzed using Typhoon Trio and ImageQuant TL system (63005583; GE healthcare Life Sciences). Densitometry analysis of intracellular IL-1β protein was normalized to GAPDH in each sample.
For cell signaling experiments, stimulated macrophages were extracted using Pierce NE-PER nuclear and cytoplasmic extraction kit (78833; Pierce) with the addition of Halt protease and phosphatase inhibitor cocktail. The nuclear fractions were then resolved by 12% SDS-PAGE and electroblotted onto PVDF membrane. Membranes were blocked with Pierce protein-free (TBS) blocking buffer (37570; Pierce) for 1 h at room temperature. The membranes were probed with primary cell signaling antibodies for 3 h at room temperature and with secondary antibodies for 1.5 h at room temperature. Then the proteins were detected immediately. After protein detection, the antibodies were removed from membrane by incubating with Amresco gentle review stripping buffer (N552; Amresco) for 30 min at room temperature, and the membranes were probed against

primary β-actin antibody (C-4; sc-47778; Santa Cruz) overnight at 4 °C. The membranes were then incubated with secondary antibodies for 1 h at room temperature. Proteins were detected and analyzed using Typhoon Trio and ImageQuant TL system. Densitometry analysis of phosphorylated proteins was normalized to β-actin in each sample. The primary antibodies used were phospho-p44/42 MAPK (Thr202/Tyr204) (4377S; Cell Signaling Technology), phospho-p38 MAPK (Thr180/Tyr182) (4511S; Cell Signaling Technology), phospho-SAPK/JNK (Thr183/Tyr185) (9251S; Cell Signaling Technology), and phospho-IKappaB-alpha (Ser32) (2859S; Cell Signaling Technology). The secondary antibodies used were DyLight 488 conjugate Goat anti-Rabbit IgG antibody and ECL Plex Goat anti-Mouse IgG-Cy5 antibody.

4.2.8 *Statistics*

All data are expressed as means ± standard error of the mean (SEM). Each biological replicate was a result of an individual activation paired with a control. Each biological replicate was performed on different days. Fold differences were calculated by comparing hCLCA1's domains-activated macrophage to its paired macrophage control. The normality and variance tests were done using Shaprio-Wilk test and Levene's test respectively, and the fold difference values of RT-qPCR and IL-1β western blot densitometry were analyzed using ANOVA following Tukey's Honestly-Significant-Difference Test[306]. For cell signaling western blot densitometry, the fold differences values were analyzed using Kruskal-Wallis one-way analysis of variance test with Conover-Inman test. All western blot data was normalized to the appropriate controls, and each western blot was a result of an individual biological replicate. Significance was determined at $p < 0.05$.

Table 4.1. Primers used for cloning.

Human CLCA1 PCR Primers for cloning	
Primer Name	**Primer Sequence (5′ → 3′)**
F.P. LIC-N1-hCLCA1	TAC TTC CAA TCC AAT GCA ATG GGC CCC TTC AAG
F.P. LIC-N240-hCLCA1	TAC TTC CAA TCC AAT GCA GCC CAG CAC GTG GAC
F.P. LIC-N643-hCLCA1	TAC TTC CAA TCC AAT GCA CTG GAC AAT GGA GCC
R.P. LIC-C544-hCLCA1	TTA TCC ACT TCC AAT GGC CTA CTT ATC GAC CAC AAA
R.P. LIC-C915-hCLCA1	TTA TCC ACT TCC AAT GGC TCA GGC GAT ACT CAG

Table 4.2. Primers used for sequencing.

Human CLCA1 PCR Primers for sequencing	
Primer Name	**Primer Sequence (5′ → 3′)**
F.P. 306-hCLCA1	ATA TAA AAA TGC AGA CGT GCT GGT CGC CGA AA
R.P. 475-hCLCA1	GCA GAG TAT GGG CCC CAG GGC CGA GCT TTT G
F.P. 900-hCLCA1	CTT CAG CCT GCT GCA GAT CGG GCA GCG GAT C
F.P. 1522-hCLCA1	GGC ACA GTG ATC GTC GAC TCA ACT GTG GGA
F.P. 2215-hCLCA1	GGG TCC TTT GTG GCT TCT GAC GTG CCA AAC GC

Table 4.3. Purification protocols for different constructs.

Construct name	Purification protocol
HMT	Ni^{2+} charged resin column → Amylose column → Dialyzed overnight with buffer D → anion exchange column
Hydrolase domain (N1-C273)	Ni^{2+} charged resin column → Amylose column → Dialyzed overnight with buffer D → anion exchange column → Size exclusion chromatography column
Hydrolase-VWA domain (N1-C544)	Ni^{2+} charged resin column → Amylose column → Dialyzed overnight with buffer D → anion exchange column → Size exclusion chromatography column
VWA domain (N240-C544)	Ni^{2+} charged resin column → Amylose column → Size exclusion chromatography column
FN3 domain (N643-C915)	Ni^{2+} charged resin column → Amylose column → Dialyzed overnight with buffer D → anion exchange column → Size exclusion chromatography column

Table 4.4. Primers used in RT-qPCR experiments.

	Human qPCR Primers	
Gene Name	**Forward Primers (5′ → 3′)**	**Reverse Primers (5′ → 3′)**
GAPDH	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG
TNF-α	TGCTGCACTTTGGAGTGATCG	TGCTACAACATGGGCTACAGG
IL-12a	CAGTGGAGGCCTGTTTACCATTG	TACTACTAAGGCACAGGGCCATC
IL-8	TCTCTTGGCAGCCTTCCTGATTTC	ATTTCTGTGTTGGCGCAGTGTG
IL-1β	GCTGATGGCCCTAAACAGATG	TGTAGTGGTGGTCGGAGATTC
IL-6	AGCCACTCACCTCTTCAGAAC	GTGCCTCTTTGCTGCTTTCAC
IL-10	AAGCTGAGAACCAAGACCCAGACA	AAAGGCATTCTTCACCTGCTCCAC

4.3 Results

4.3.1 *Purification of hCLCA1 domain proteins*

DNAs of different hCLCA1 domains were subcloned into a pET28 HMT vector: hydrolase domain (N1-C273), von Willebrand factor type A (VWA) domain (N240-C544), hydrolase-VWA domain (N1-C544), and fibronectin type III (FN3) domain (N643-C915) (Figure 4.1), and each of the clones contains a hexahistidine-tag (His_6-tag) and a maltose binding protein (MBP) at the N-terminal end of the sequence. The sequences of the clones were confirmed prior to expression and purification using fast protein liquid chromatography with the following purification columns: Ni^{2+}-charged resin column, amylose column, anion exchange column, and size exclusion chromatography column (Table 4.2). The eluted proteins were analyzed by coomassie stained gel and the fractions containing the desired proteins were further polished by endotoxin removal procedures (Figure 4.2). After that, the purity of the proteins was confirmed by coomassie stained gel (Figure 4.3).

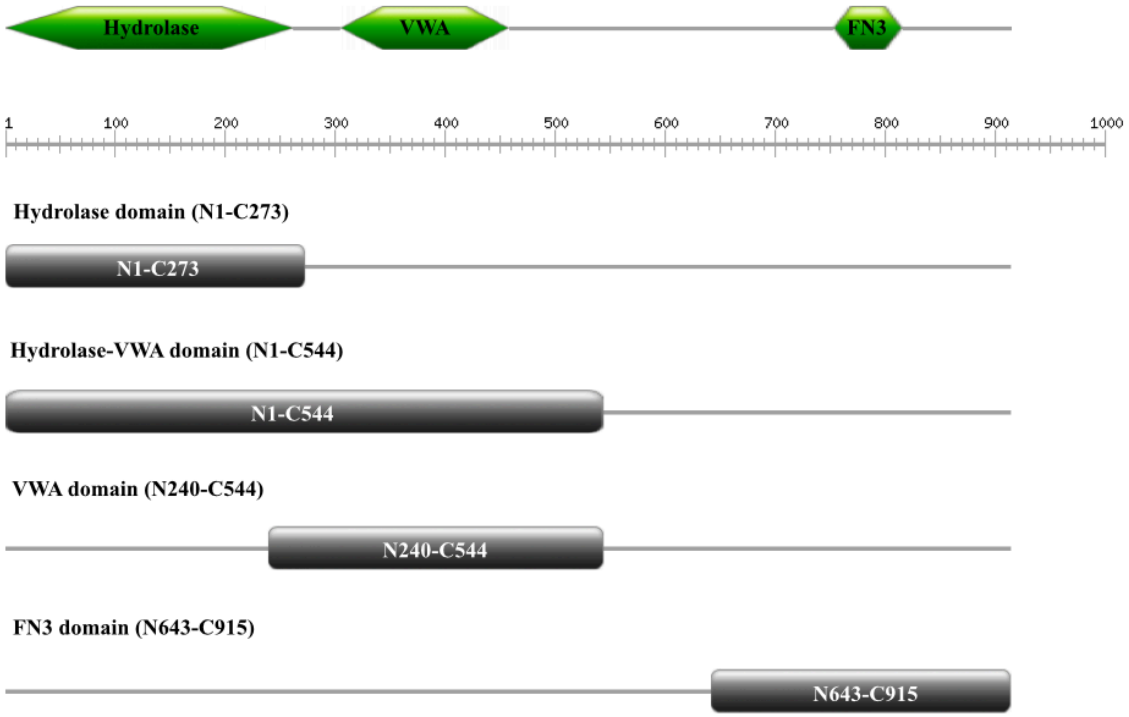

Figure 4.1. hCLCA1 domain DNA constructs design. Four DNA constructs were designed to capture different domains of hCLCA1: N1-C273 contains the hydrolase domain, N1-C544 contains the hydrolase and VWA domain, N240-C544 contains the VWA domain, and N643-C915 contains the FN3 domain.

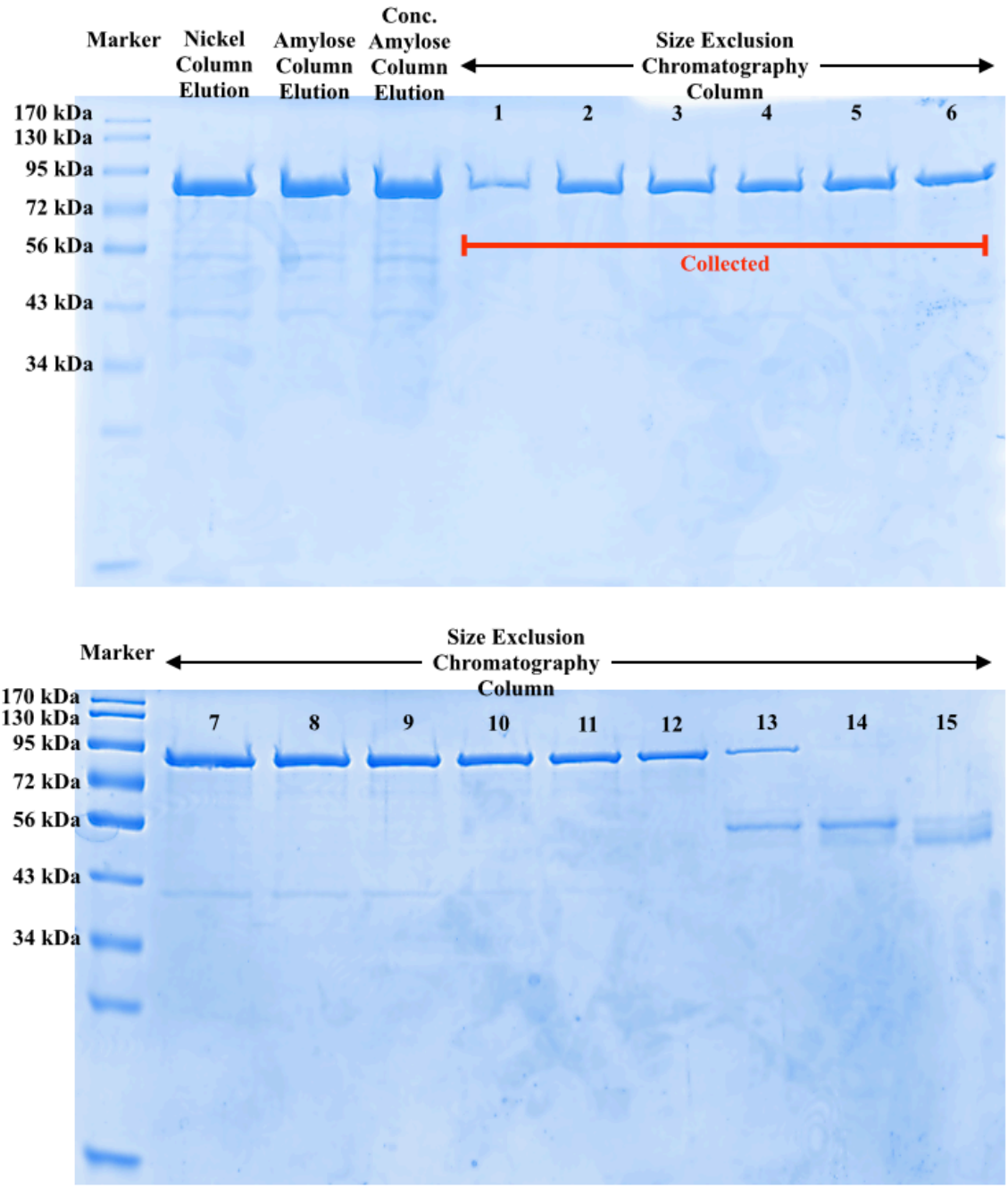

Figure 4.2. Representative coomassie stained gel showing the purification process of a recombinant protein. The recombinant protein (VWA domain in this case) was initially purified with a nickel column, followed by an amylose column and a size exclusion chromatography column. Only the fractions containing pure protein (fraction # 1-6) from size exclusion chromatography column were collected for subsequent experiments.

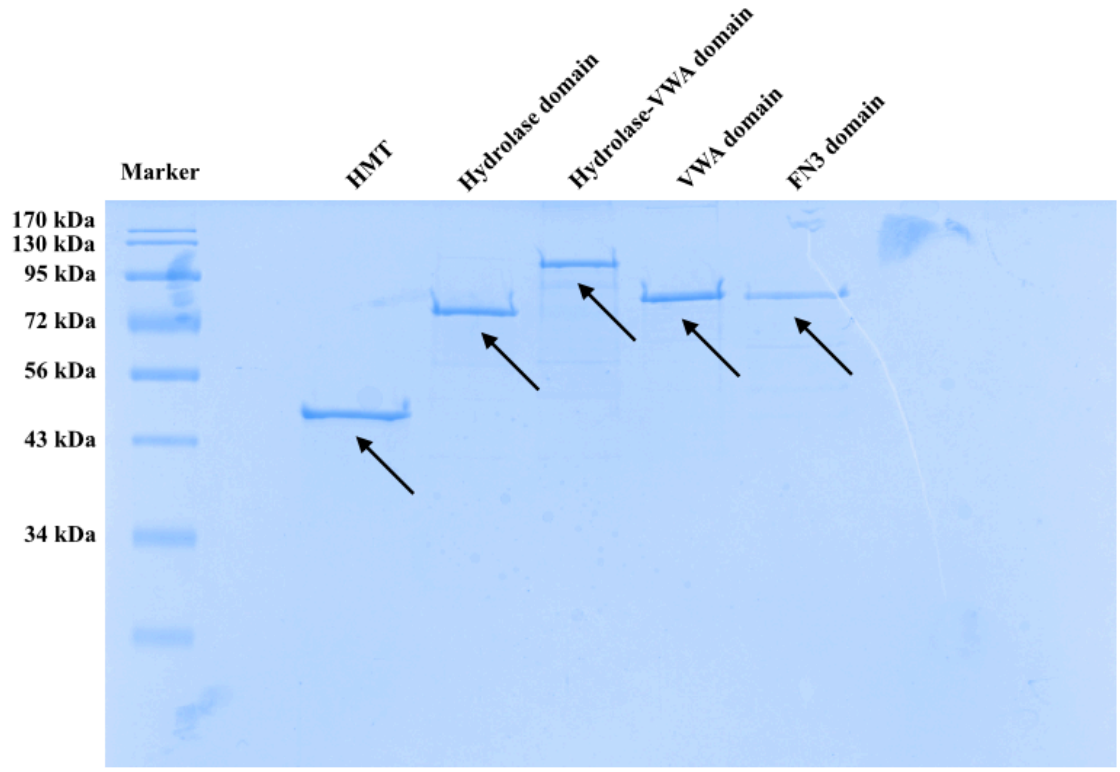

Figure 4.3. Representative coomassie stained gel showing the purity of different hCLCA1 domain proteins. Different recombinant proteins were expressed and purified, and subsequently analyzed on a coomassie stained gel. The recombinant proteins were loaded from left to right: HMT vector (negative control), hydrolase domain (N1-C273), hydrolase-VWA domain (N1-C544), VWA domain (N240-C544), and FN3 domain (N643-C915).

4.3.2 Activation of the U-937 macrophages with hCLCA1 domains

Activation of macrophages by hCLCA1 domains was investigated using the U-937 macrophage cell line treated with 5 μg/mL of purified hCLCA1 domain proteins for 48 h. Macrophage activation was assessed by expression of the pro-inflammatory cytokines TNF-α, IL-8, IL-1β, IL-6, and IL-10. The mRNA expression of IL-1β in VWA-treated macrophages had a 2.08±0.19 fold increase over the control (Figure 4.4). The IL-1β mRNA expression in VWA-treated macrophages was also significantly over the HMT-treated macrophages. HMT protein was used as an additional control because all purified hCLCA1's domain proteins contain a His_6-tag and a MBP at the N-terminal end of the sequence. Beside VWA domain, none of the other hCLCA1's domain proteins elicit any pro-inflammatory response in macrophages.

The increase in IL-1β mRNA expression was translated into an elevated IL-1β protein levels. Western blot and densitometry analysis showed that VWA-treated macrophages had a 2.38±0.18 fold increase in IL-1β protein expression over the control (Figure 4.5). Also, the IL-1β protein expression of VWA-treated macrophages was also significantly increased over the HMT-treated macrophages. Similar to the mRNA experiment, none of the other hCLCA1's domain proteins induce IL-1β protein expression compared to the control.

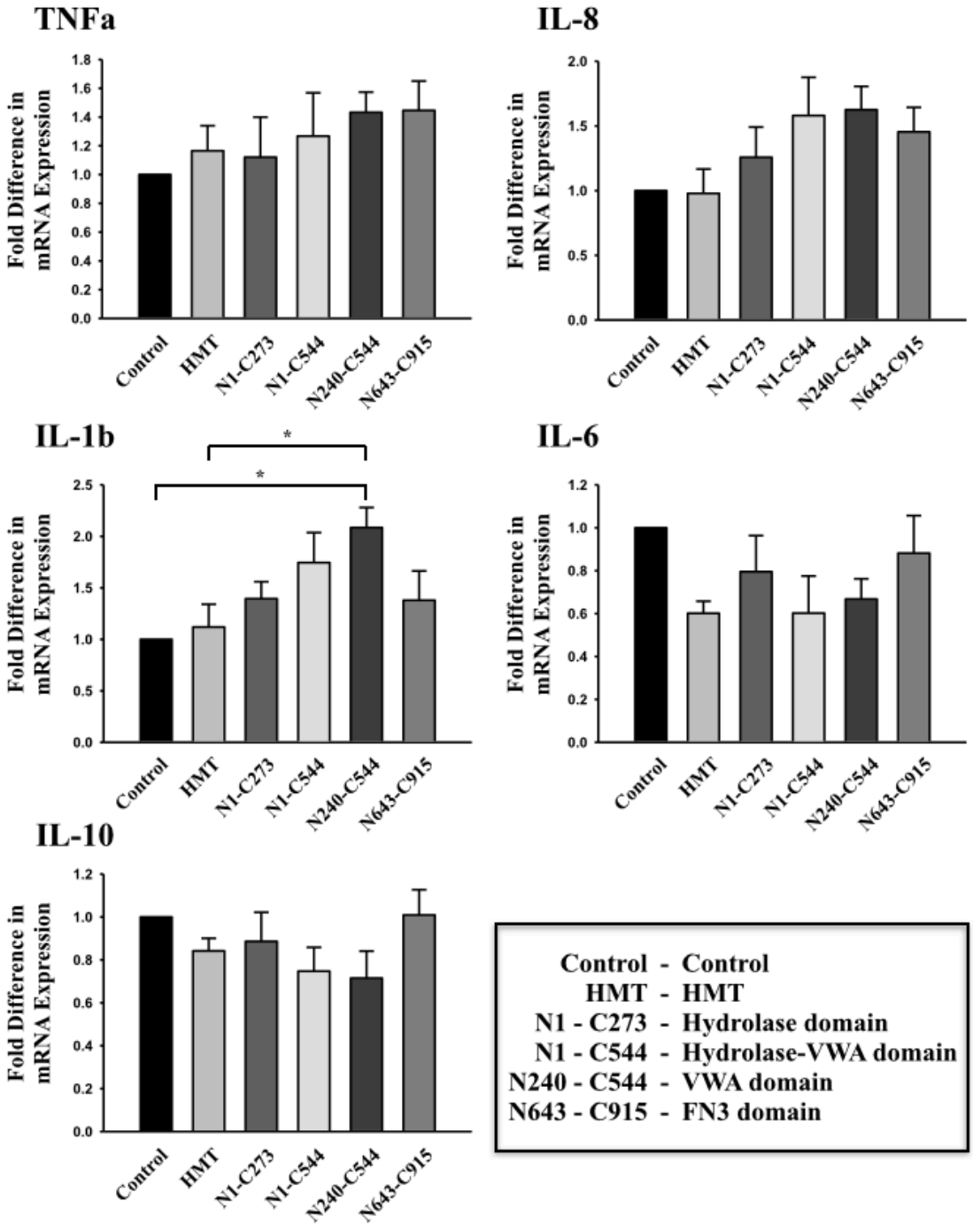

Figure 4.4. Effect on cytokine expression of macrophages exposed to 5 µg/mL purified hCLCA1 domains. U-937 macrophages were treated for 48 h using 5 µg/mL of different purified hCLCA1 domain proteins. TNF-α, IL-8, IL-1β, IL-6, and IL-10 were quantified by their mRNA expression using RT-qPCR. N240-C544 (VWA domain) has a 2.08 ± 0.19 fold increase over the control in IL-1β mRNA expression level. The fold difference of each sample was compared against the control. Results were the means of 5 samples ± SEM. Significant fold differences from corresponding control values are indicated by * ($p < 0.05$), ** ($p < 0.005$) and *** ($p < 0.001$).

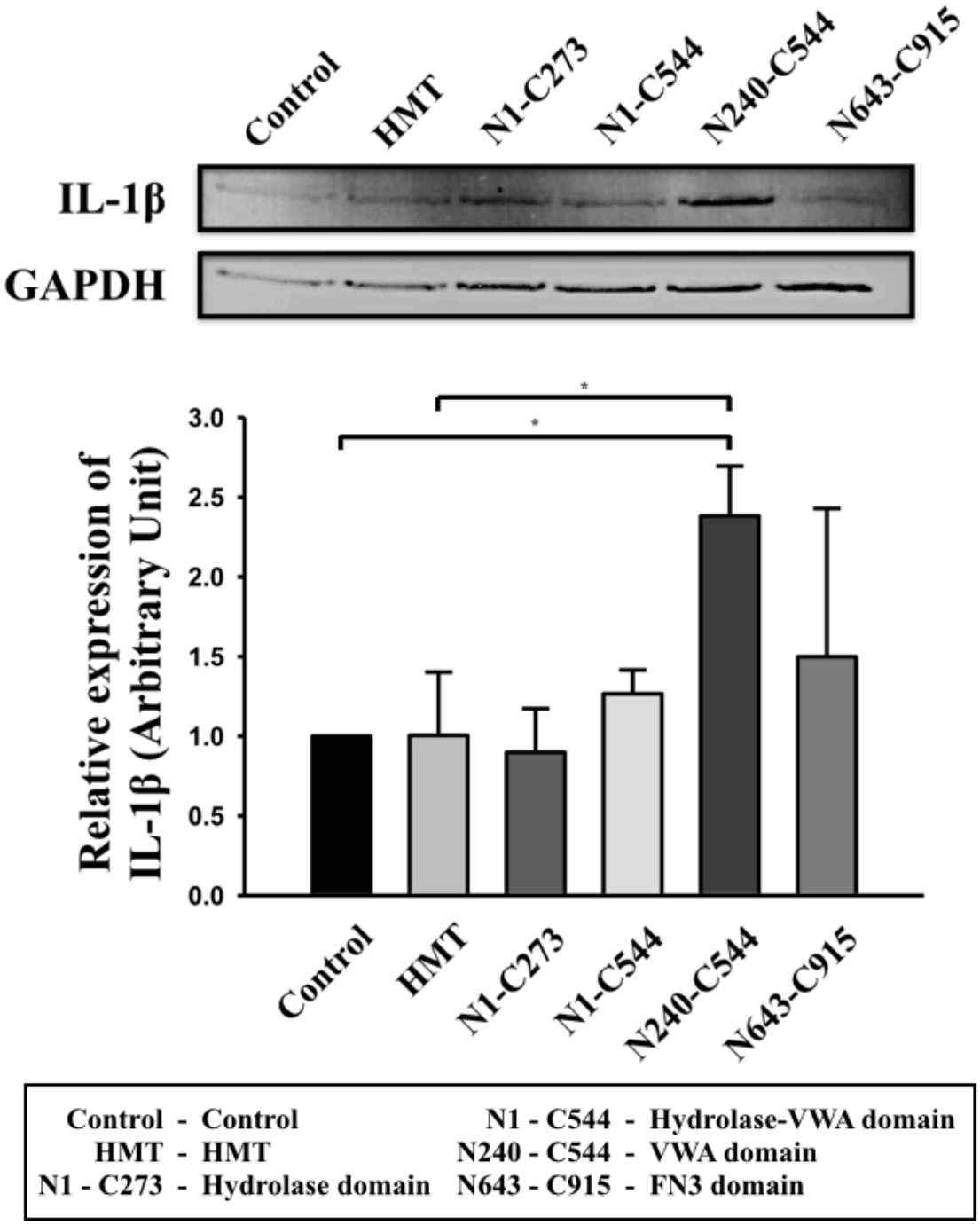

Figure 4.5. VWA domain (N240-C544) of hCLCA1 increased IL-1β protein expression level. U-937 macrophages were treated for 48 h using 5 μg/mL of different purified hCLCA1 domain proteins. Western blot and densitometry showed that 5 μg/mL VWA domain treatments induced a 2.38 ± 0.18 folds increase in IL-1β protein expression, while other hCLCA1 domains treatment showed no effect. The fold difference of each sample was compared against the control. Results were the means of 3 samples ± SEM. Significant fold differences from corresponding control values are indicated by * ($p < 0.05$), ** ($p < 0.005$) and *** ($p < 0.001$).

4.3.3 *Phosphorylation of MAPKs and NF-κB pathways by VWA domain*

To determine the signal transduction pathway by which VWA domain is involved in this induction, macrophages were treated with 5 μg/mL HMT protein or VWA domain protein for 2, 4, 6, or 12 h. Nuclear fraction proteins were extracted and analyzed using western blot against phospho-specific antibodies involved in MAPK and NF-κB pathway. MAPK pathway was tested with p-p38, p-ERK, and p-JNK antibodies, while NF-κB pathway was tested with p-IκB-α antibody. To validate each antibody, western blot was performed on nuclear fraction proteins extracted from LPS-induced macrophages (Figure 4.6).

Western blot and densitometry analysis showed that VWA domain significantly induced the phosphorylation of IκB-α (Figure 4.7), p38 (Figure 4.8), and ERK (Figure 4.9) in macrophages over the HMT protein-activated macrophages, while JNK was neither detected in HMT-treated nor VWA-treated macrophages (data not shown). Generally, increased phosphorylation was observed with increased incubation duration of VWA domain, this indicates a potential positive feedback mechanism was involved to perpetuate the inflammatory phenotype of macrophages.

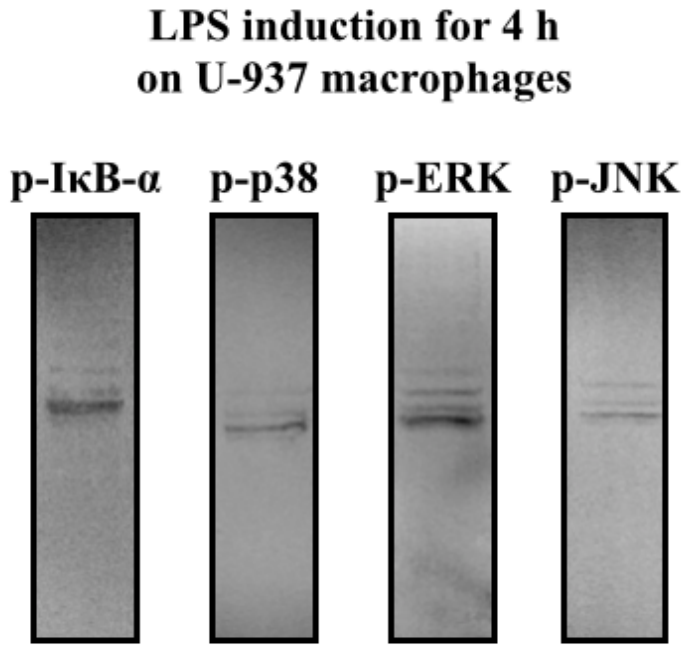

Figure 4.6. Validation of phosphor-specific antibodies with LPS-induced macrophages. Macrophages were activated with 1 μg/mL LPS for 4 h before protein isolation. Nuclear fraction proteins were analyzed with western blot against different phosphor-specific antibodies: p-IκB-α, p-p38, p-ERK, and p-JNK. Following LPS induction, the phosphorylated proteins were successfully detected by the phosphor-specific antibodies.

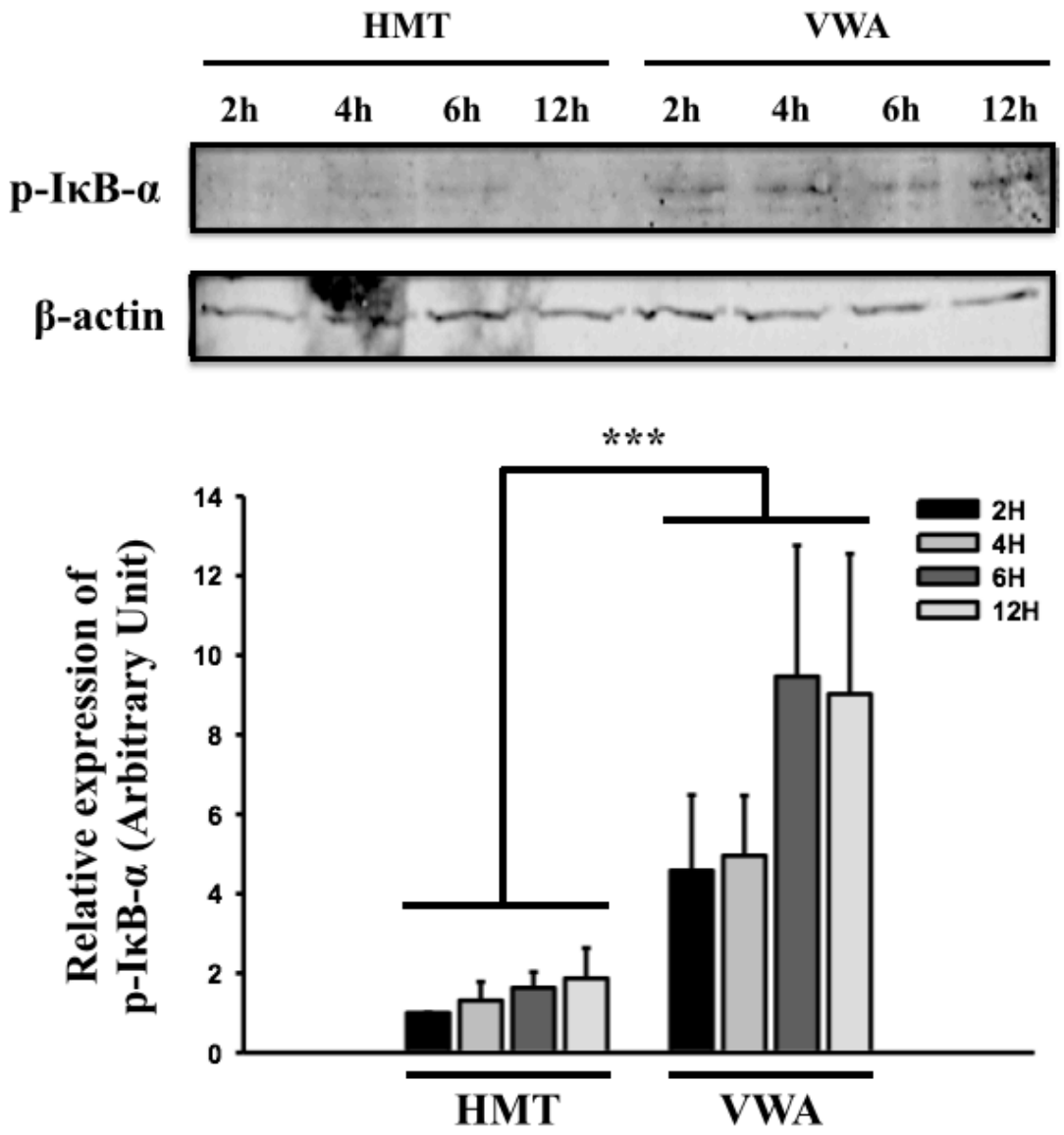

Figure 4.7. Phosphorylation of IκB-α with VWA domain treatment. U-937 macrophages were treated for 2, 4, 6, or 12 h with 5 μg/mL of HMT protein or VWA domain protein. Western blot and densitometry showed that VWA domain significantly induced the phosphorylation of IκB-α. The fold difference of each sample was compared against HMT-treated macrophage at 1 h. Results were the means of 3 samples ± SEM. Significant fold differences from corresponding control values are indicated by * ($p < 0.05$), ** ($p < 0.005$) and *** ($p < 0.001$).

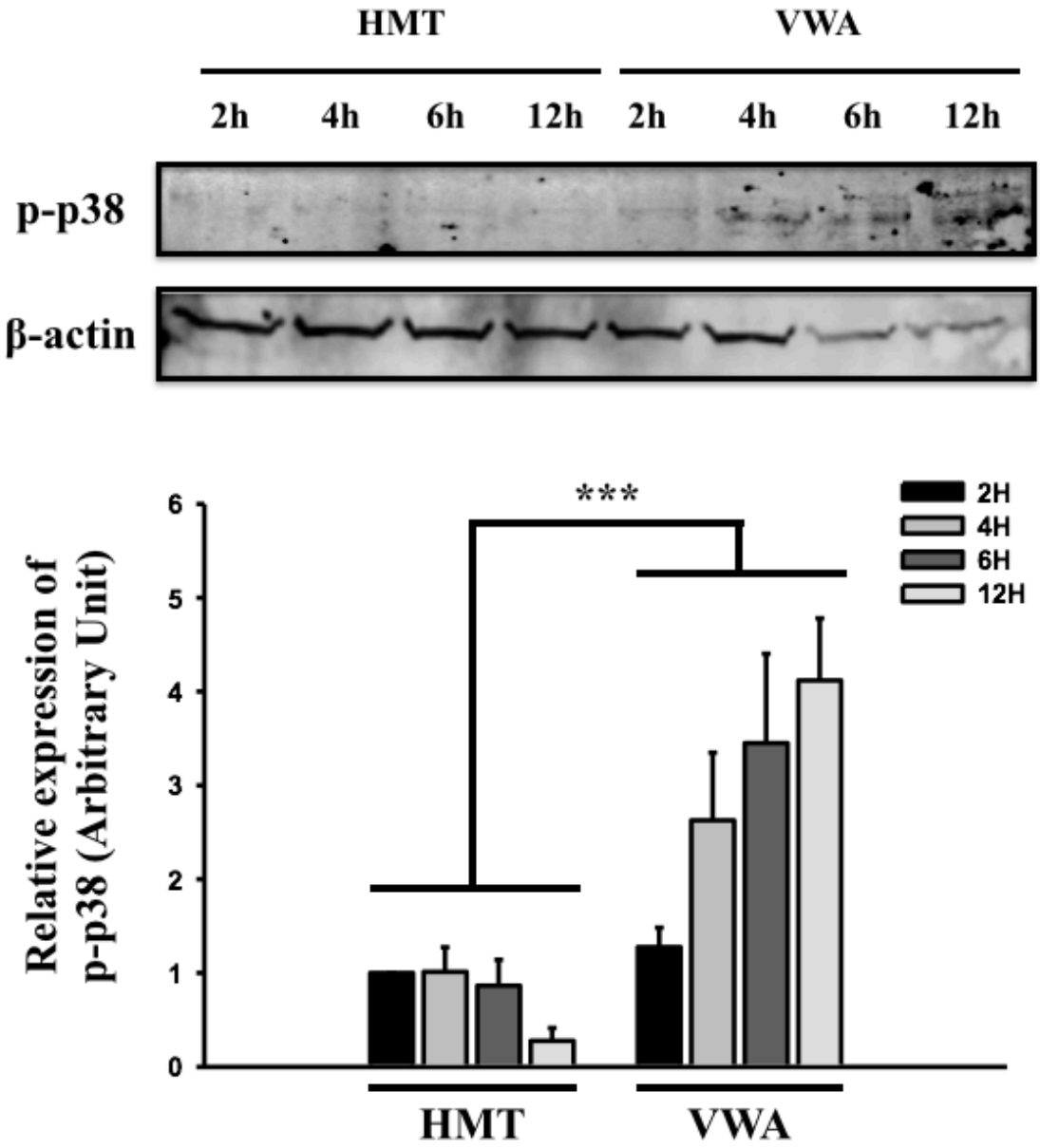

Figure 4.8. Phosphorylation of p38 with VWA domain treatment. U-937 macrophages were treated for 2, 4, 6, or 12 h with 5 μg/mL of HMT protein or VWA domain protein. Western blot and densitometry showed that VWA domain significantly induced the phosphorylation of p38. The fold difference of each sample was compared against HMT-treated macrophage at 1 h. Results were the means of 3 samples ± SEM. Significant fold differences from corresponding control values are indicated by * ($p < 0.05$), ** ($p < 0.005$) and *** ($p < 0.001$).

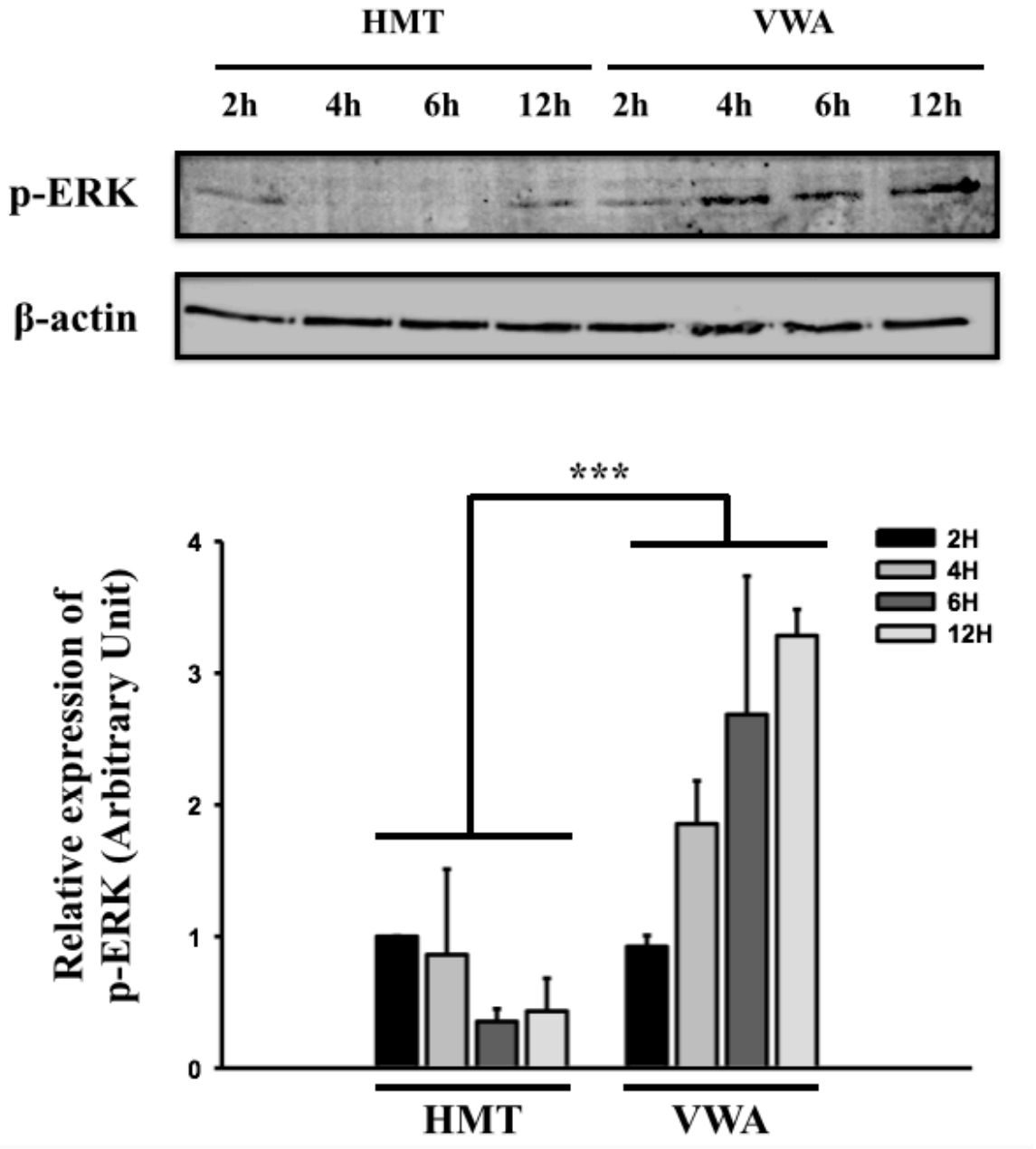

Figure 4.9. Phosphorylation of ERK with VWA domain treatment. U-937 macrophages were treated for 2, 4, 6, or 12 h with 5 μg/mL of HMT protein or VWA domain protein. Western blot and densitometry showed that VWA domain significantly induced the phosphorylation of ERK. The fold difference of each sample was compared against HMT-treated macrophage at 1 h. Results were the means of 3 samples ± SEM. Significant fold differences from corresponding control values are indicated by * ($p < 0.05$), ** ($p < 0.005$) and *** ($p < 0.001$).

4.4 Discussion

In our previous study, we have demonstrated that secreted hCLCA1 has the capability to induce pro-inflammatory cytokines in macrophages[45]. Recent studies have further support our proposed novel function of hCLCA1 as a signaling molecule[37, 41, 100]. In the present study, we identified the functional domain of hCLCA1 responsible for macrophage activation, and we also described a signal transduction pathway involved in this activation. The results demonstrate that the von Willebrand factor type A domain of hCLCA1 is responsible for IL-1β induction in macrophages, and this induction correlated with the activation of NF-κB and MAPK signaling pathway.

4.4.1 *Purification of hCLCA1 domain proteins*

Initially, we subcloned different segments of the hCLCA1 gene into the HMT vector. HMT vector was chosen because the expressed fusion protein will contain a His_6-tag and a MBP at the N-terminus of the protein, which will allow for protein purification using fast protein liquid chromatography. MBP is thought to act as a general molecular chaperone to assist protein folding[318, 319]. This was confirmed as we observed protein degradation of hydrolase domain protein (N1-C273) after we cleaved and removed the protein tags using tobacco Etch Virus (TEV) protease. Although previous studies have reported that MBP-tag in fusion protein does not reduce bioactivity[320, 321], we cannot exclude the possibility that it might create conformation hindrance on the binding site of the protein. After proteins were purified, endotoxin was removed from the proteins using a Triton X-114 phase separation technique. Using Triton X-114, Adam et al. showed a 100-fold endotoxin reduction in 2 steps, however, 50% loss in bioactivity of the protein was also observed[322]. In our experiment, a 3-step protocol was required to reduce the endotoxin level below 0.01 EU/mL, which potentially results in a greater loss of bioactivity in the protein. Our results demonstrated that the activation of macrophages was solely due to the VWA domain and not to LPS contamination because phosphorylation was observed only in ERK, p38, NF-κB, but not JNK. On the other hand, phosphorylation of ERK, p38, JNK, and NF-κB was observed in LPS-induced macrophages (Figure 4.6).

4.4.2 *Macrophage activation by von Willebrand factor type A domain*

hCLCA1 domain proteins were used to activate U-937 macrophages. A preliminary concentration response experiment was initially performed in which macrophages were treated with 1 μg/mL or 5 μg/mL of proteins. However, only 5 μg/mL proteins were able to induce inflammatory response. In our previous study, ~150 pg/mL of immuno-purified hCLCA1 was sufficient to induce macrophage activation, while another study reported the physiological concentration of hCLCA1 in IL-13-induced NHBE cells was ~0.23 μg/mL[41]. A seemingly high dose of hCLCA1 domain proteins required for macrophage activation in this study might possibly be attributed to reduced bioactivity of the proteins. As mentioned above, the conformation hindrance imposed by the MBP tag and treatment with Triton-X114 could drastically reduce the bioactivity of the proteins. In addition, secreted hCLCA1 has been shown to be a highly glycosylated protein[7, 21], and glycosylation is known to modulate the structure and function of signaling molecules[65-67]. The lack of glycosylation on hCLCA1 domain proteins using the *E. coli* expression system might have a detrimental effect on its bioactivity. Together, a dramatically reduced bioactivity might be explained, thus providing a possible explanation for the high concentration of proteins required to activate macrophages.

Of all the protein constructs tested (HMT, hydrolase domain, hydrolase-VWA domain, VWA and FN3 domain), only VWA domain protein elicited an inflammatory response in macrophages, and the response was limited to IL-1β (Figure 4.4). Western blot and densitometry analysis showed that the increase in IL-1β mRNA expression was translated into an elevated protein level (Figure 4.5). This result coincides with our previous findings, in which IL-1β was the most induced cytokine activated by immuno-purified hCLCA1 (Figure 3.11)[45]. It is important to note that although hydrolase-VWA domain did not induce any significant inflammatory response, it did induce the second highest IL-1β and IL-8 mRNA expression (Figure 4.4). This might be attributed to the structural conformation of the protein that prevents the accessibility of binding site for protein interaction.
The VWA domain is a well-studied domain involved in cell adhesion, in extracellular matrix proteins, and in integrin receptors[71-73]. The VWA domain of hCLCA1 contains three metal-

ion-dependent adhesion site (MIDAS) motifs. Previous studies have demonstrated that the MIDAS motif in the α2δ subunit of the voltage gated calcium channel is essential for its function in calcium channel trafficking and modulation[74-77]. A recent study has demonstrated that secreted hCLCA1 increased calcium-activated chloride channel conductance[37], similar to how the α2δ subunit of the voltage gated calcium channel modulates its function[74]. From these studies, it is possible that the MIDAS motifs in hCLCA1's VWA domain are the active sites that are responsible for macrophage activation.

4.4.3 *Activation of MAPKs and NF-κB pathways by VWA domain*

To investigate the cell signaling pathway which VWA induced in macrophages, we performed western blot for selected phospho-specific proteins involved in NF-κB and MAPK. These two pathways were chosen because activation of these pathways result in IL-1β expression[234, 323-326]. From our western blot analysis, we observed an increase in phosphorylation of IκB-α, p38 and ERK with VWA domain incubation (Figure 4.7, Figure 4.8, and Figure 4.9). However, phosphorylation of JNK was not observed with VWA domain incubation.

It has been well documented that both activation of NF-κB pathway and phosphorylation of ERK result in IL-1β expression[234, 252, 326, 327], and phosphorylation of p38 contributes to the activation of NF-κB pathway[328-330]. Interestingly, one group has reported that phosphorylation of p38 activates the NF-κB pathway, resulting in an up-regulation of macrophage inflammatory protein 1α (MIP-1α), a chemokine for macrophage recruitment[331]. These studies, together with our findings, partly explain how hCLCA1 modulates macrophage function. Secreted hCLCA1 interacts with macrophages, activating both ERK and p38. Phosphorylation of p38 subsequently activates NF-κB pathway and induces MIP-1α expression, recruiting more macrophages to the site of inflammation. This creates a positive feedback system in which more macrophages are available for hCLCA1 to interact with. Then, activation of NF-κB pathway and phosphorylation of ERK together, induce IL-1β expression in macrophages, thus enhancing the inflammatory response (Figure 4.10).

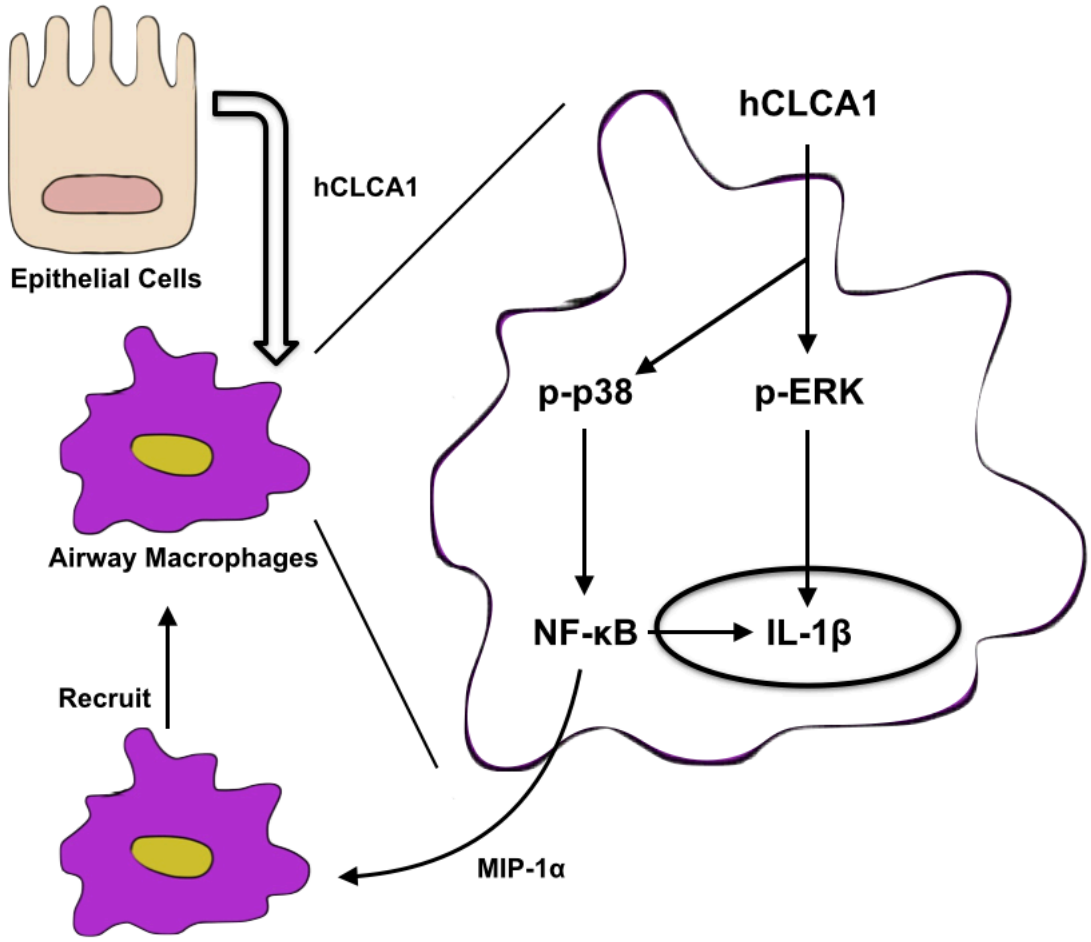

Figure 4.10. Schematic model of how hCLCA1 induces inflammatory response in airway macrophages. We hypothesized that hCLCA1 secreted from inflamed epithelial cells activates macrophages via the activation of ERK and p38, in which p38 activation leads to activation of NF-κB, and together they induce IL-1β expression. In addition, activation of NF-κB by p38 also increases the expression of MIP-1α, which recruits additional macrophages to the site of inflammation to perpetuate the innate immune response.

4.5 Conclusions

In our previous study, we have described the novel ability of secreted hCLCA1 to function as a signaling molecule that can activate airway macrophages[45]. Such ability likely has a profound impact on the immune response in the airways, where the expression of this gene is massively up-regulated during inflammation[14]. In the present study, we have identified the VWA domain of hCLCA1 as the one responsible for macrophage activation, and this correlates with the activation of NF-κB and MAPK pathways. These pathways lead to an increase in inflammatory cytokines expression. The cytokine response of the macrophages would then modify the inflammatory response, mucus secretion, and airway responsiveness in airway diseases. Studies to identify the receptor will be of great therapeutic interest because it will provide new way to reduce the severity of airway inflammation, but it is beyond the scope of this paper.

GENERAL DISCUSSION

5.1 Implication

Airway diseases such as asthma, cystic fibrosis, or chronic obstructive pulmonary diseases are one of the leading causes of morbidity and mortality worldwide. Patients suffering from these airway diseases often suffer from chronic inflammation, mucus hypersecretion, airflow obstruction, and even respiration failure or death during disease exacerbation. During airway inflammation, inflamed epithelial cells secrete increased levels of cytokines and chemokines to initiate the innate immune response. These molecules recruit inflammatory cells to the site of inflammation, and these inflammatory cells secrete additional inflammatory mediators to amplify the immune response. Although these immune responses are important to protect the host against pathogens, failure to resolve inflammation brings forth a detrimental effect. These are salient in the cases of COPD and asthma, in which persisted inflamed airways lead to chronic bronchitis, emphysema, airway remodeling, and severe airway constriction.

Beside inflammatory mediators such as cytokines and chemokines, hCLCA1 is one of the most induced proteins expressed in inflamed airways. It has a well-documented involvement in goblet cell metaplasia in patients suffering from these airway diseases. However, its role in inflammation has remained unknown. In our studies, we demonstrated that hCLCA1 has the ability to modulate the immune response in airway macrophages. We showed that secreted hCLCA1 could act as a signaling molecule to induce inflammatory response both in U-937 macrophage cell line and in primary porcine alveolar macrophages. Furthermore, we have identified VWA domain of hCLCA1 as the functional domain responsible for macrophage activation, and it is via activation of NF-κB and MAPK pathway. Our findings suggest that hCLCA1 has the potential to amplify the inflammatory response in macrophages, and this might contribute to the tissue damages associated with airway diseases.

Therefore, understanding the functional domain and mechanism can provide directions on novel treatment to control inflammation triggered by macrophages in airway disease patients, thus minimizing the severity of the diseases. It is also of great therapeutic interest because it provides a non-steroidal anti-inflammatory drugs alternative, thus eliminating possible side effects such as weight gain, insomnia, osteoporosis, etc.

5.2 Future Research

In our studies, we have identified the VWA domain of hCLCA1 to be responsible for macrophage activation. However, the key amino acid residues involved in this interaction remains unknown. Therefore, a mutation study should be carried out to identify the specific amino acid residues. Specific amino acid residues within the VWA domain determined by proteomic bioinformatics tools will be mutated into alanine through site-directed mutagenesis. The mutated proteins will be added to the macrophage to determine which mutations can attenuate macrophage activation. This information will be vital to hCLCA1 inhibitor design to ameliorate the severity of airway inflammation.

We would also like to identify the binding partners of hCLCA1 through polyHis-tagged pull down assay. HMT-fused VWA domain protein will be immobilized on a nickel column, and macrophage cell lysate will be loaded onto the column for pull-down assay. The interaction complex will be eluted and analyzed with western blot and mass spectrometry. If the interaction between hCLCA1 and its binding partner is weak, a crosslinking assay will be used instead. To confirm that the binding ligand discovered is responsible for macrophage activation, heterozygous dominant negative constructs of the ligand will be synthesized and added to the macrophage, followed by hCLCA1 activation. Then RT-qPCR or western blot will be used to evaluate cytokine expression.

In our second study, we proposed a possible mechanism by which hCLCA1 modulates the inflammatory response in macrophages through activation of the ERK and p38-dependent NF-κB pathways. To confirm this, a temporal experiment should be carried out using inhibitors targeting the ERK and p38 pathway, follow by western blot against IL-1β or IκB-α. This will allow us to definitively show which cell signaling pathway hCLCA1 initially triggers, and whether such cell signaling pathway result in inflammatory response in macrophages.

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